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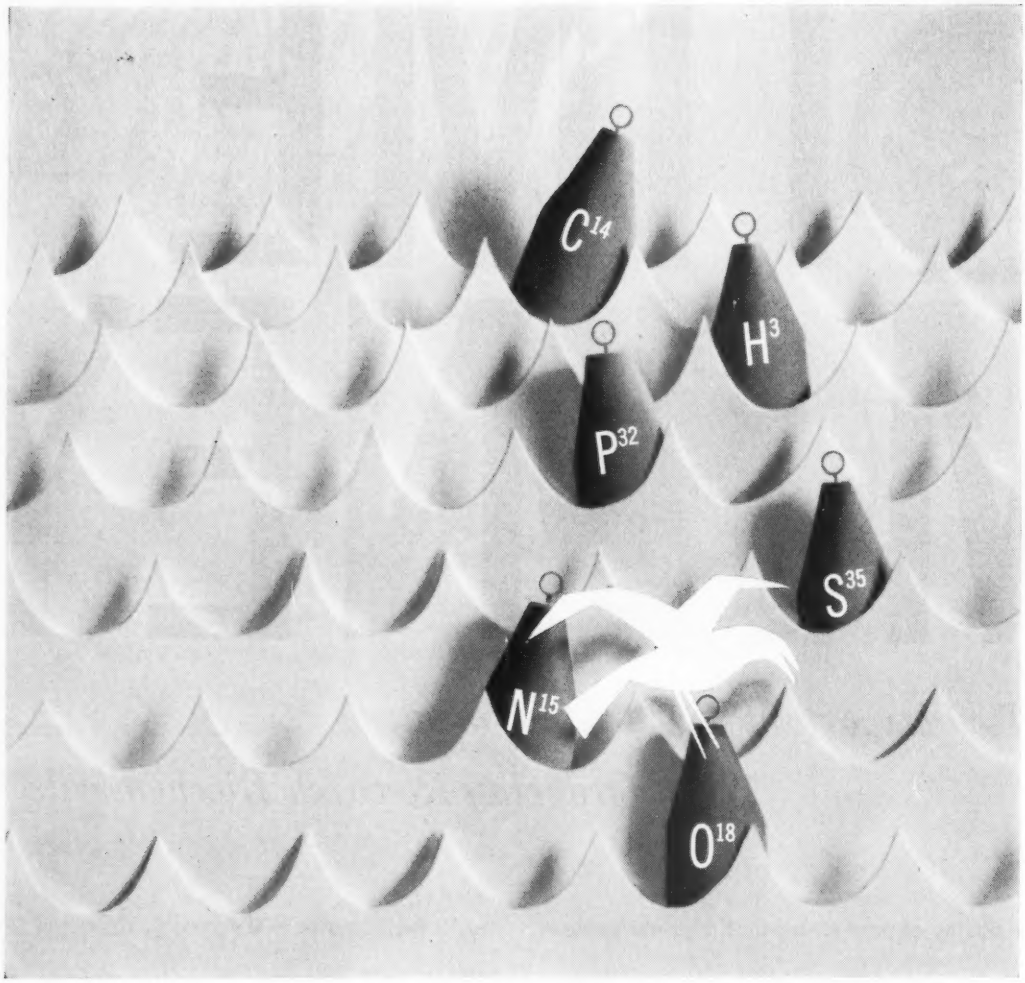

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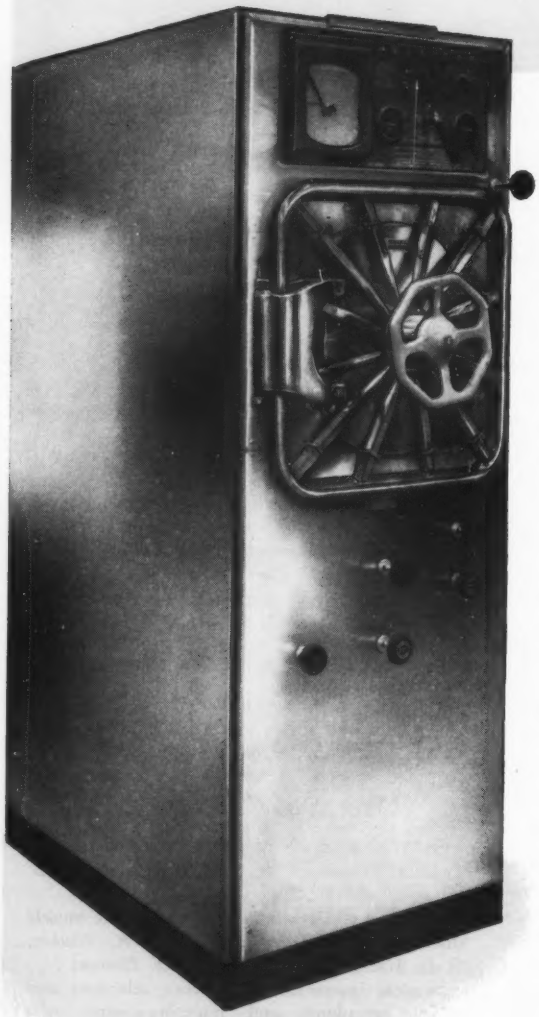

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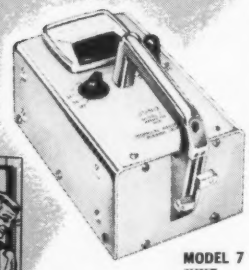

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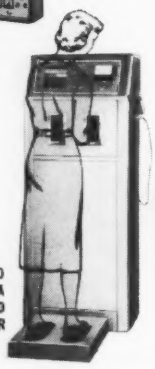

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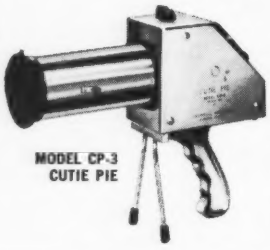

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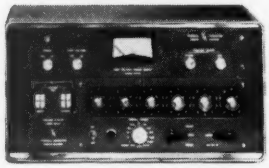

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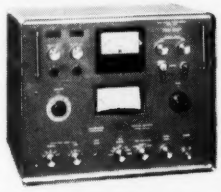

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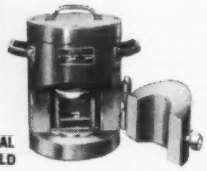

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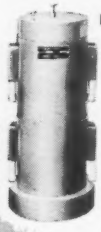

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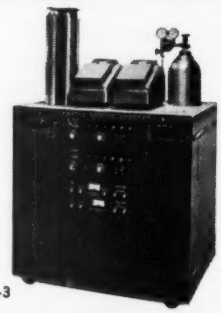

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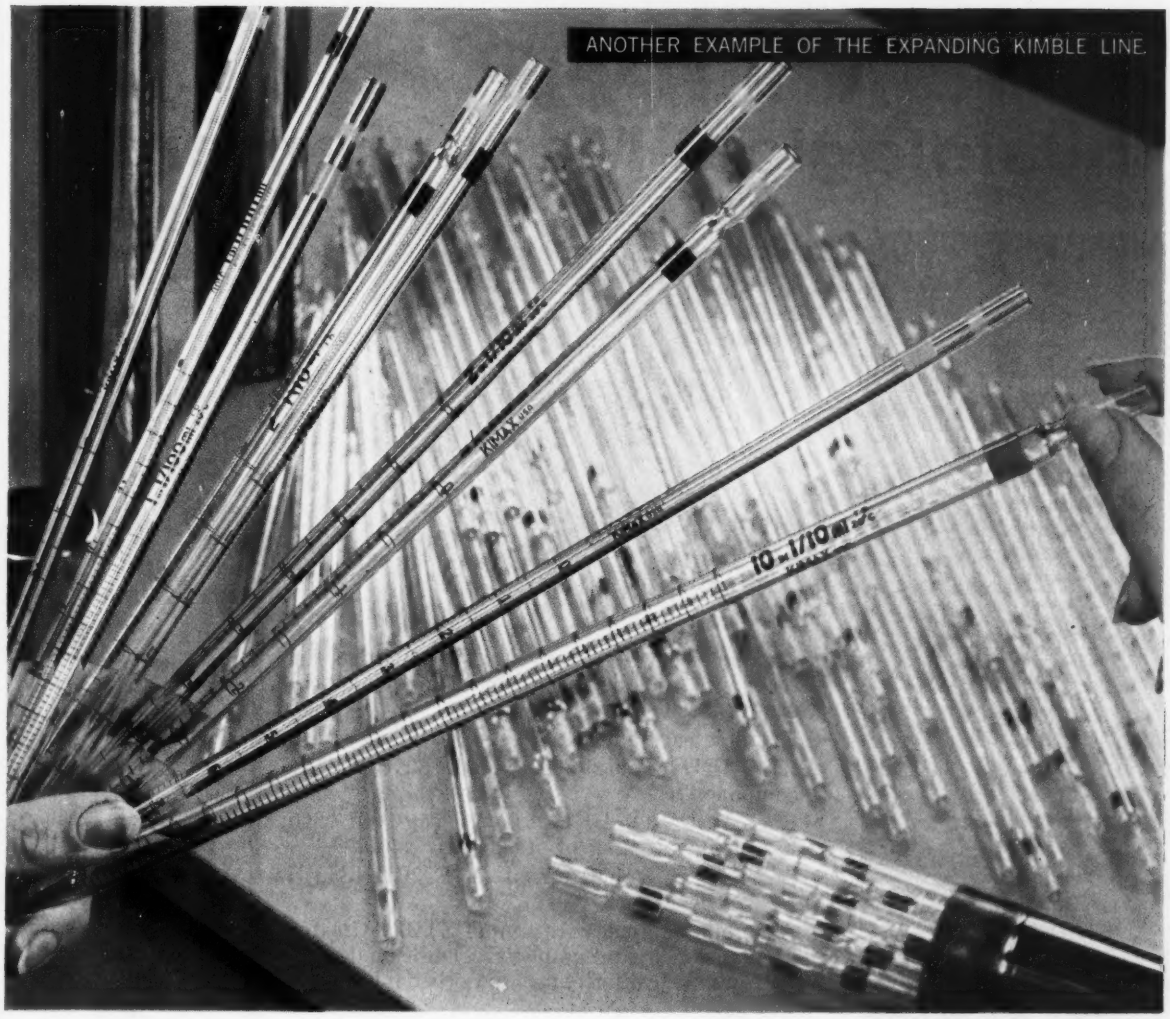

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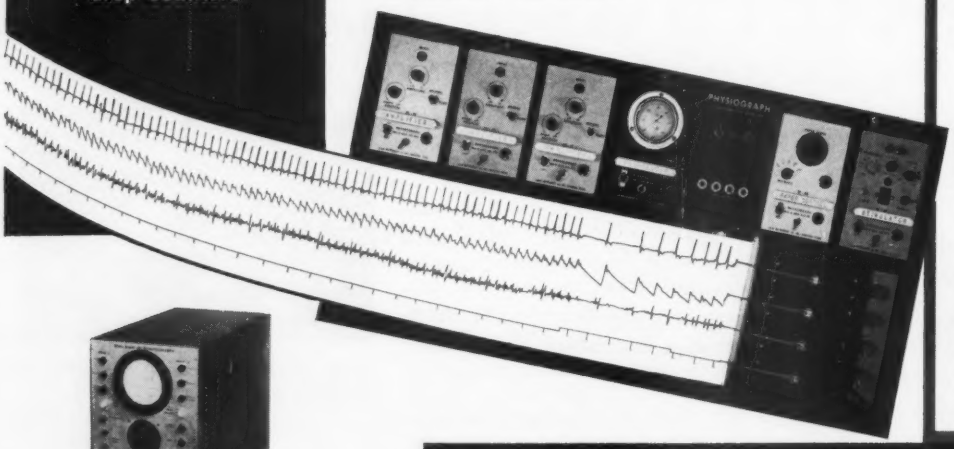

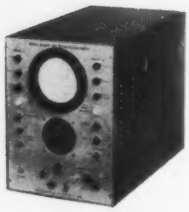

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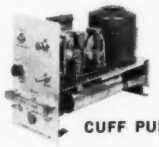

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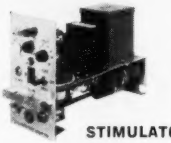

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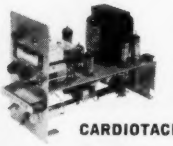

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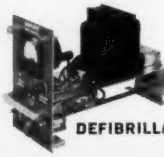

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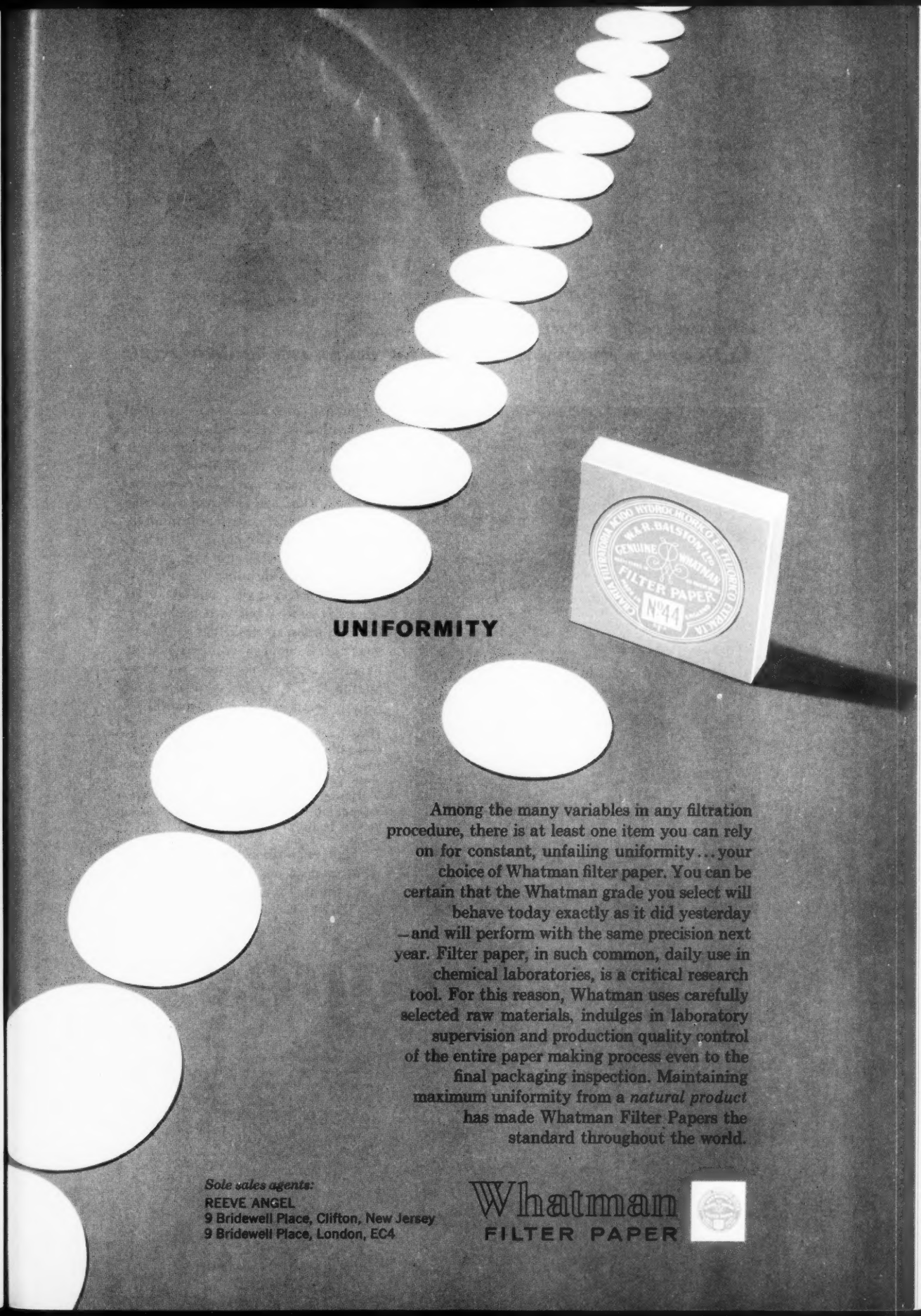

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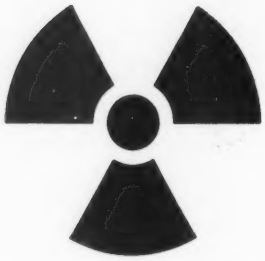

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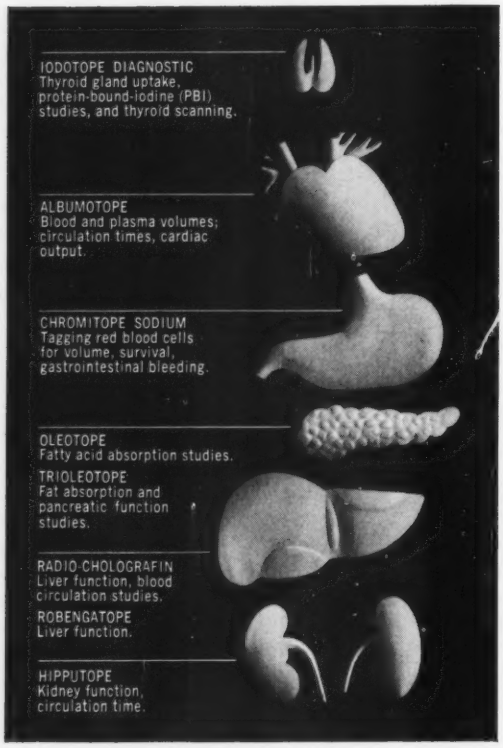

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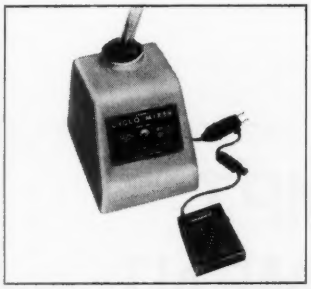

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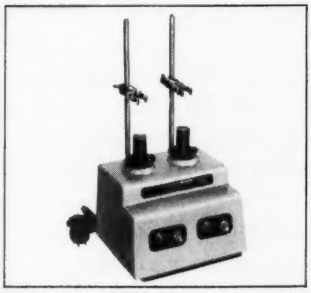

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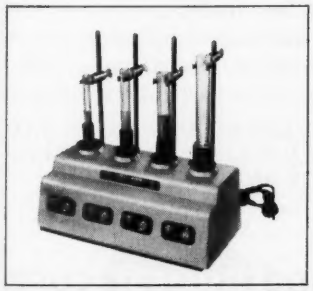

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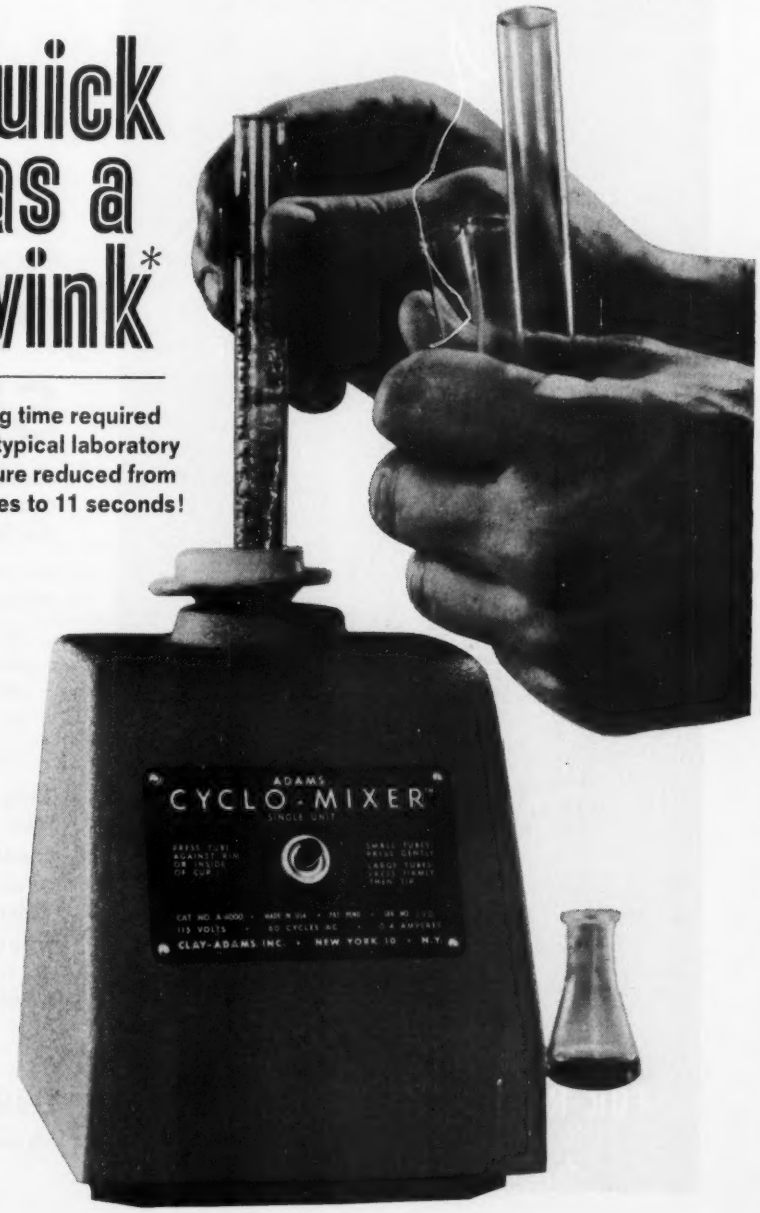

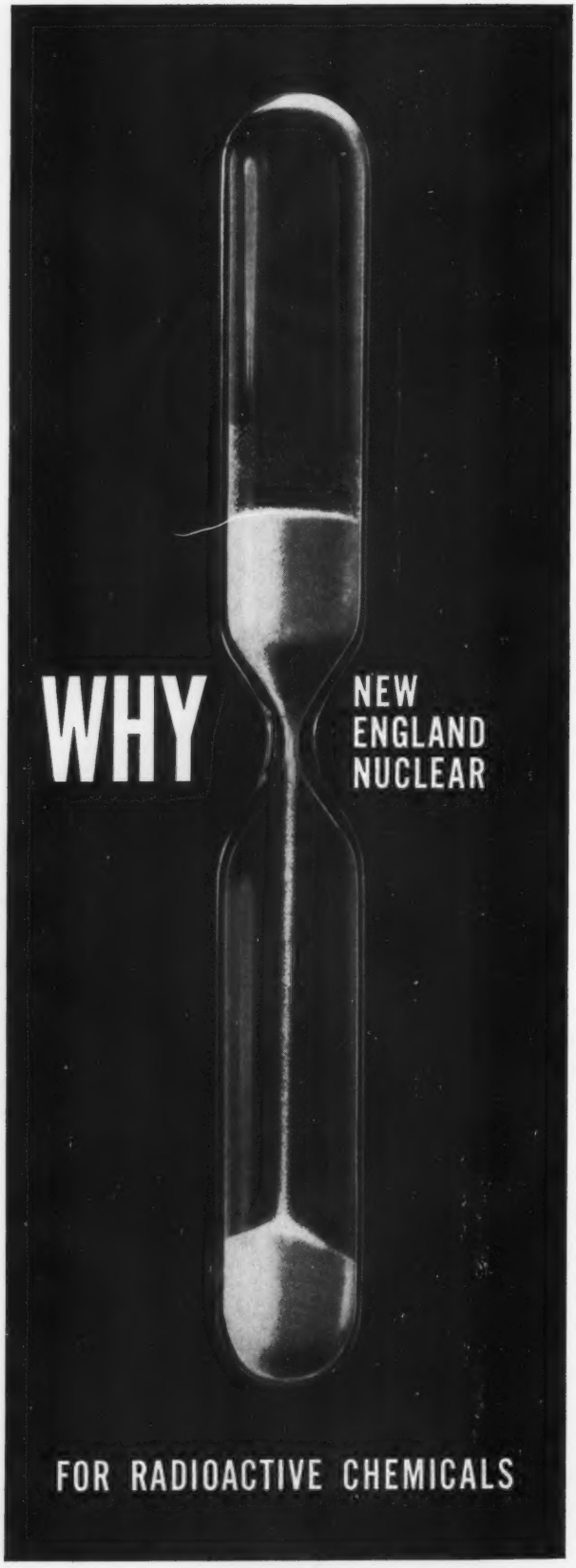

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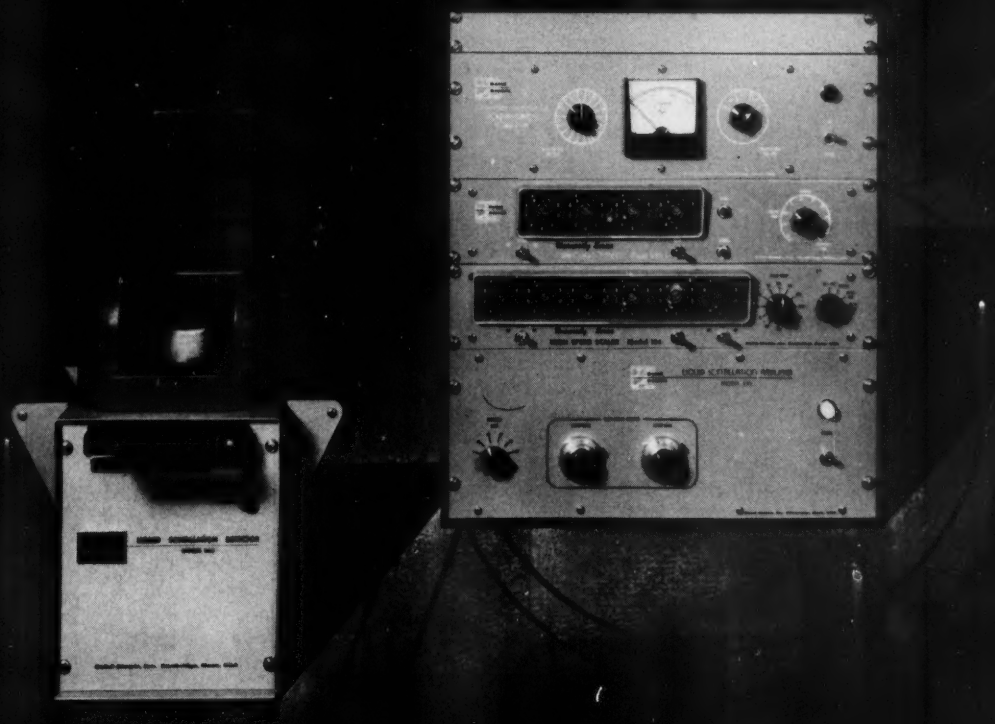

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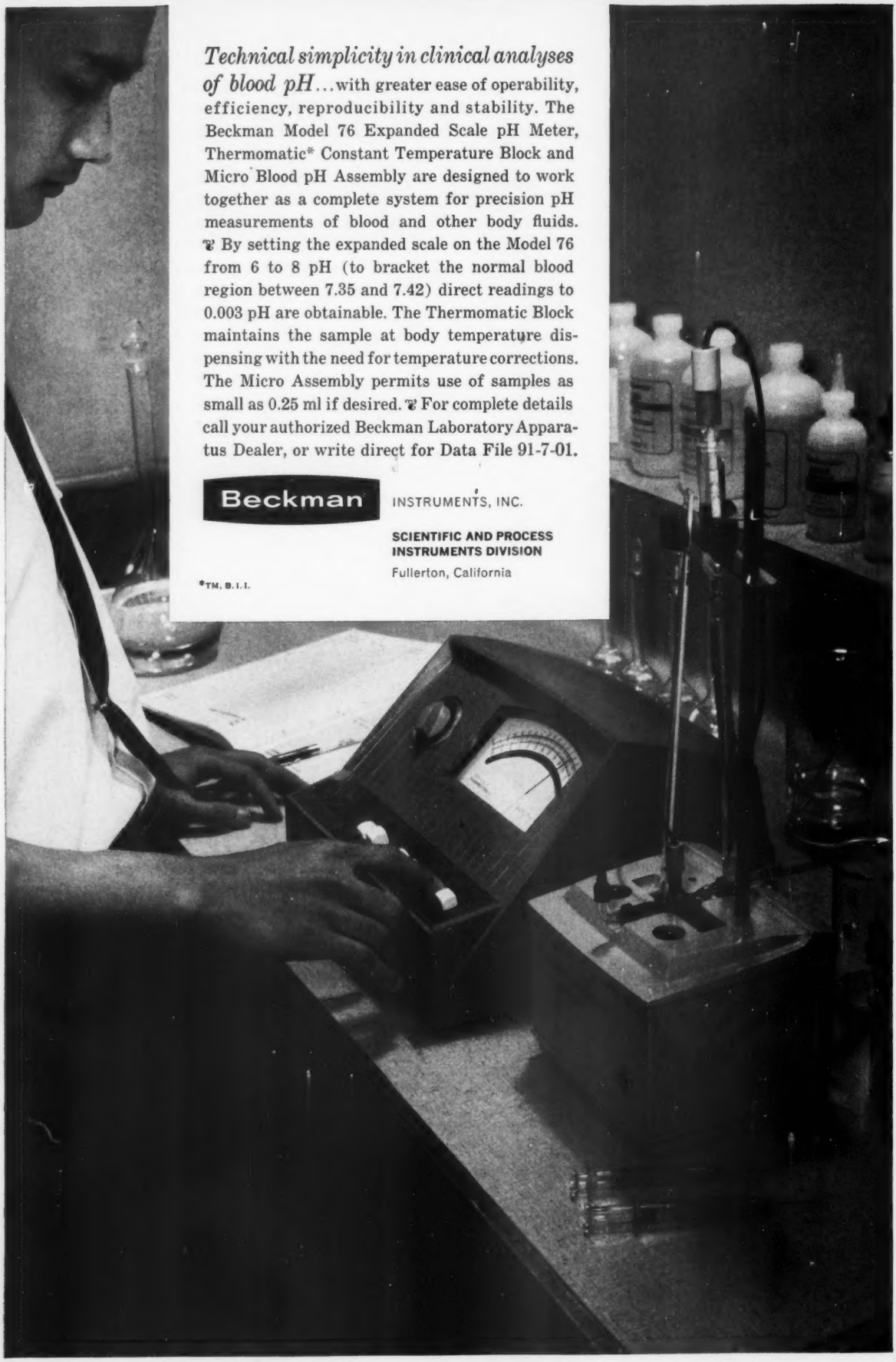

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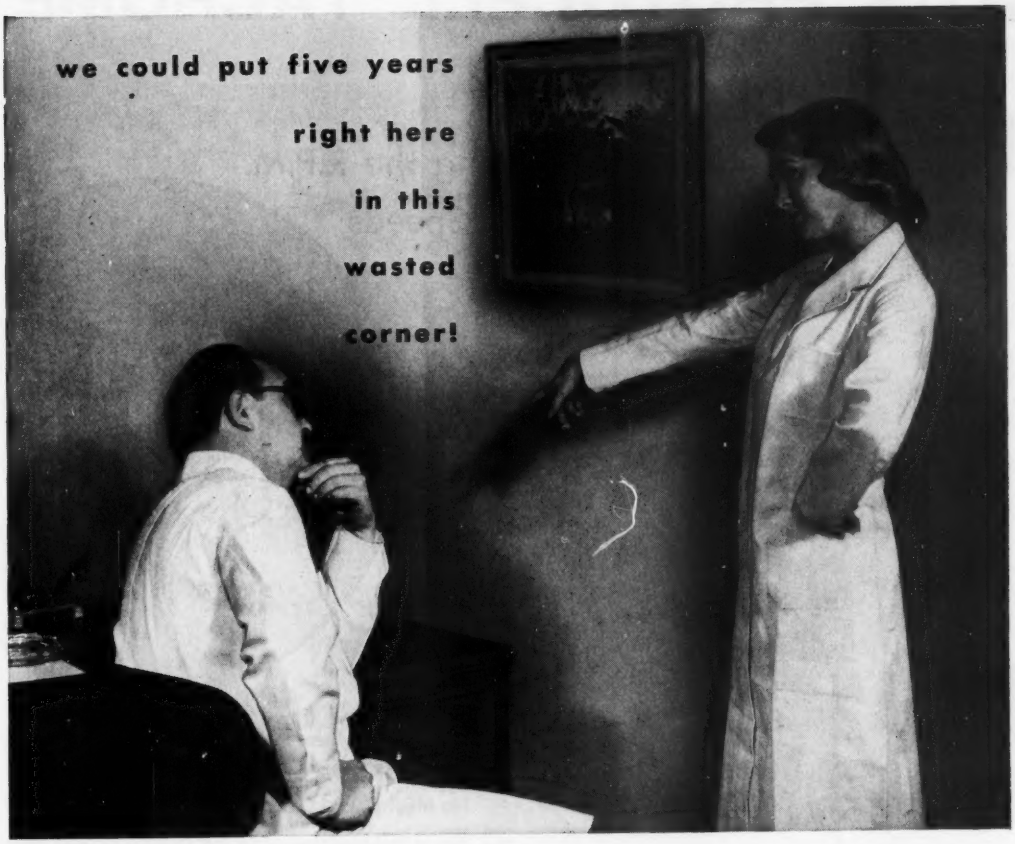

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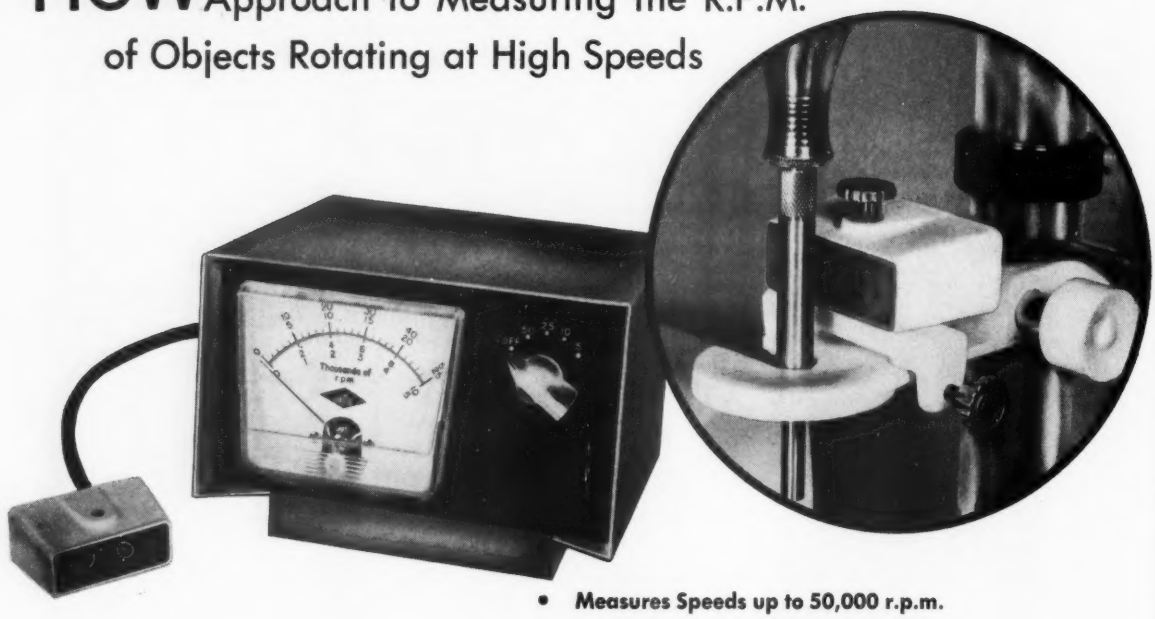

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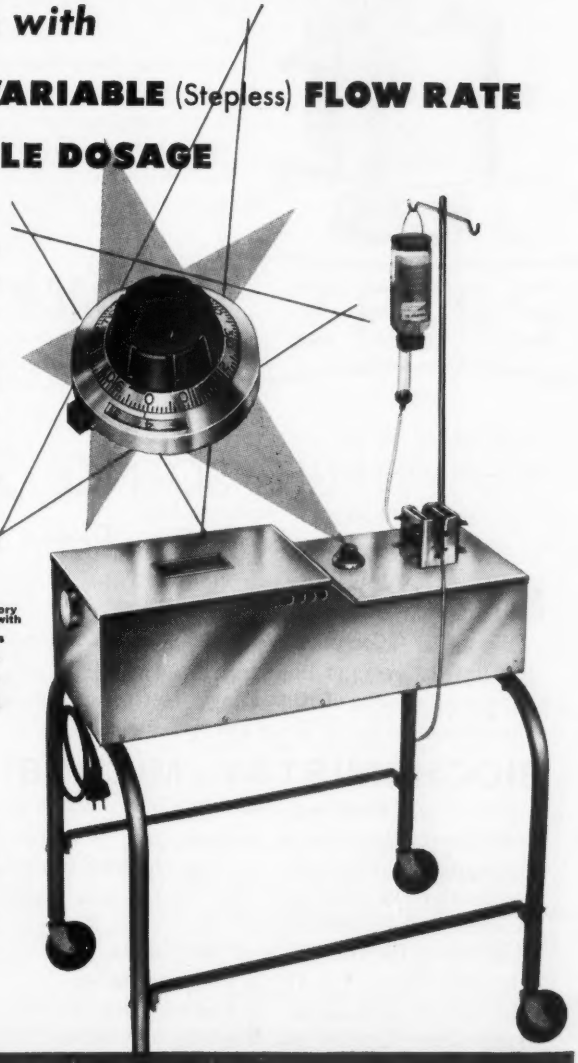

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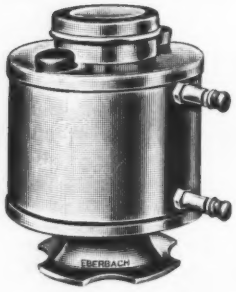

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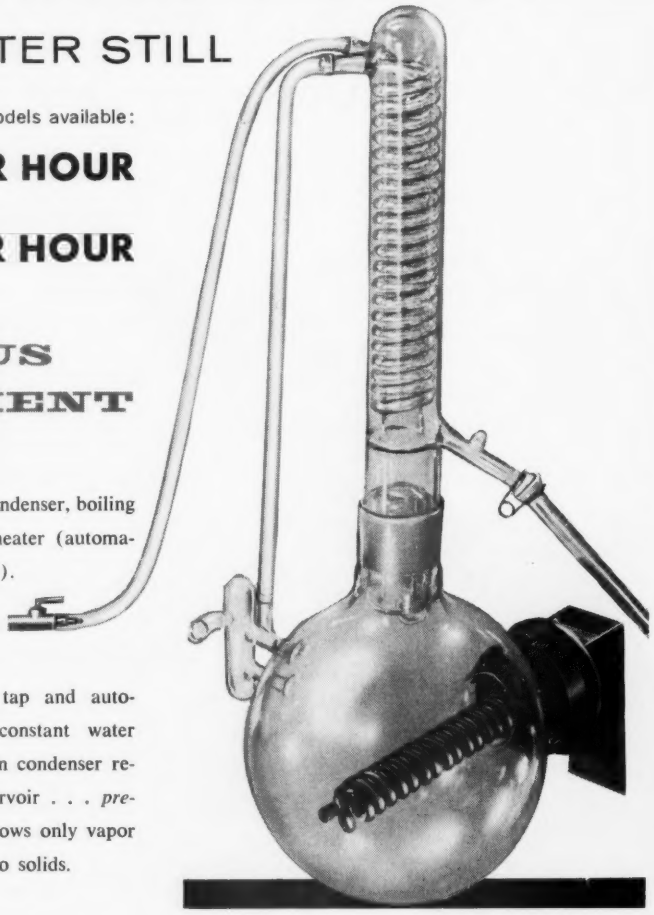

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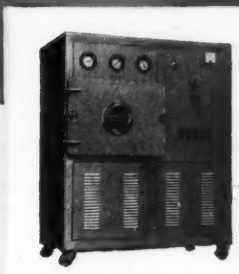

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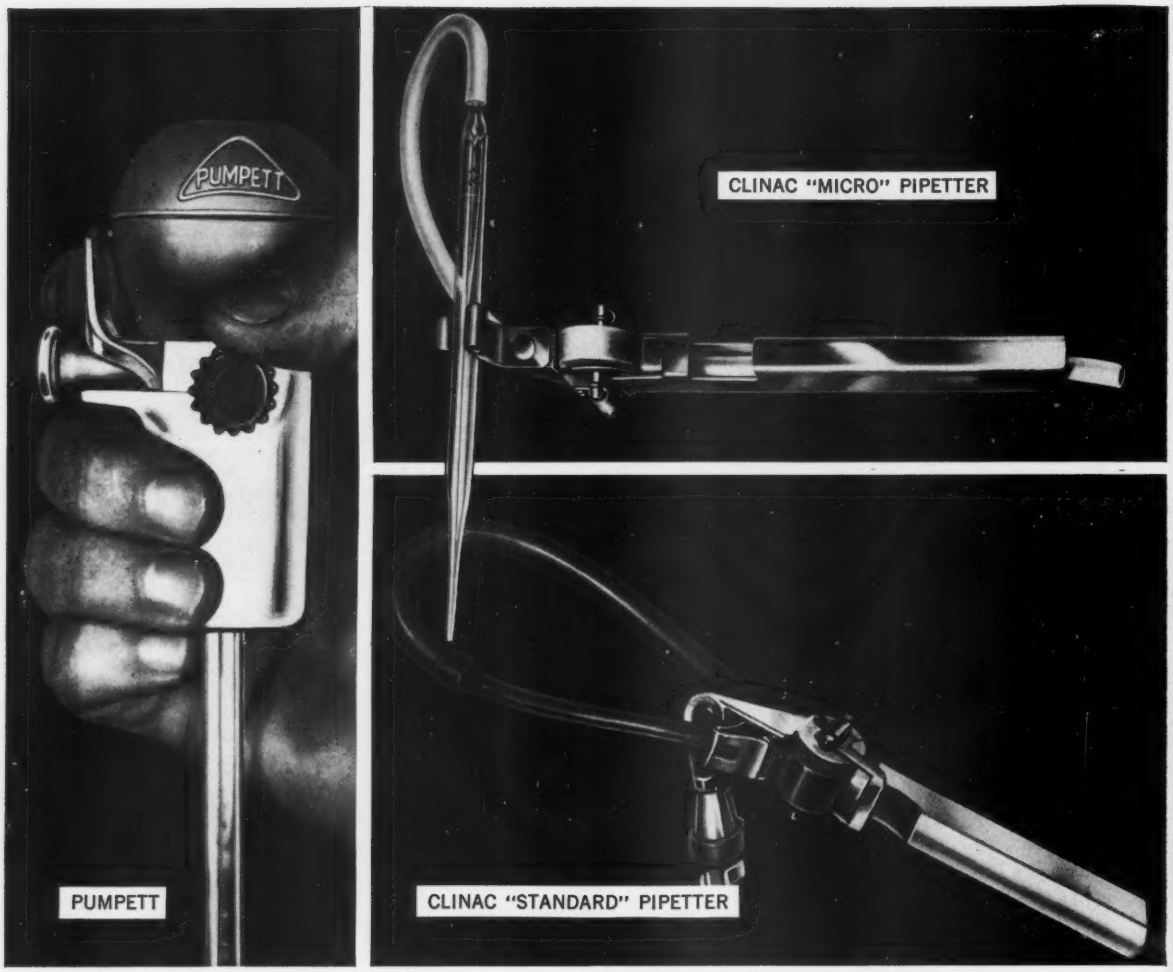

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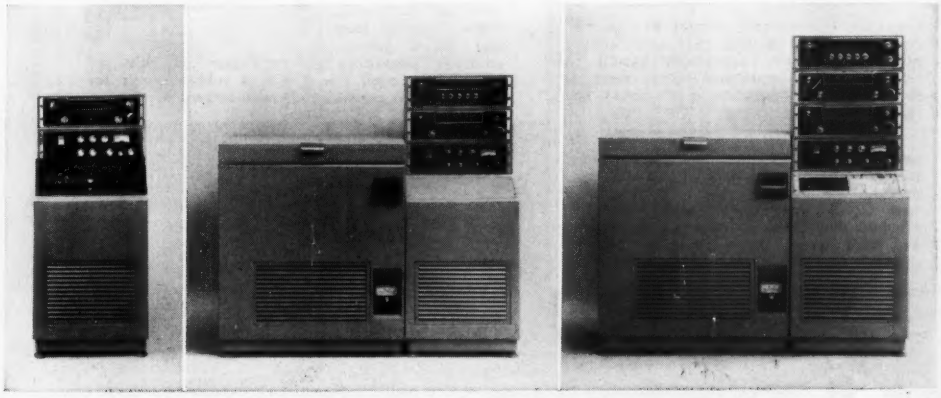
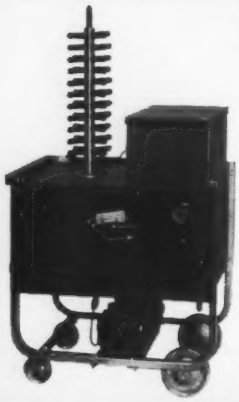
Model 701
manual operation, shown with 8250 scalcr

Model 702
manual, temperature-controlled operation, shown with 8250 scaler and 8600 timer

Model 703
automatic, temperature-controlled operation, shown with two 8250 scalers, 8600 timer and 8401 digital recorder





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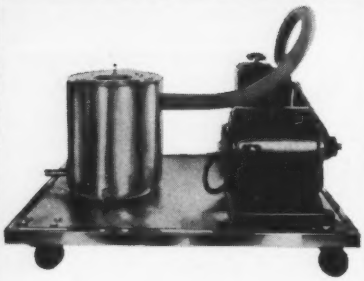

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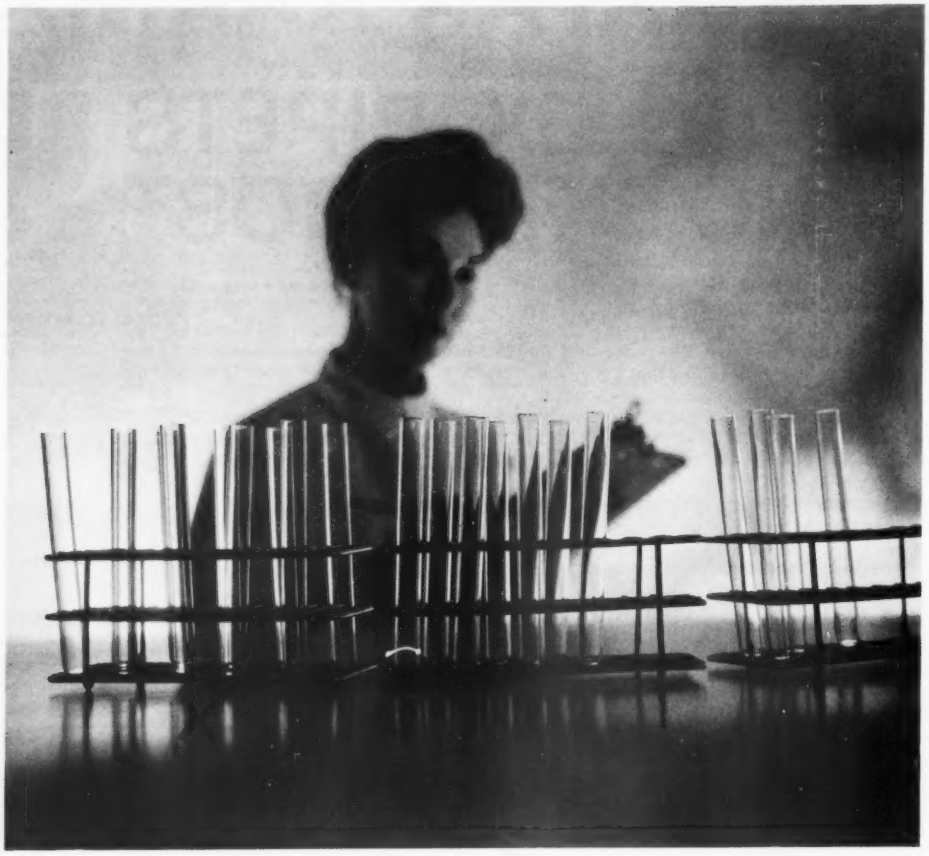

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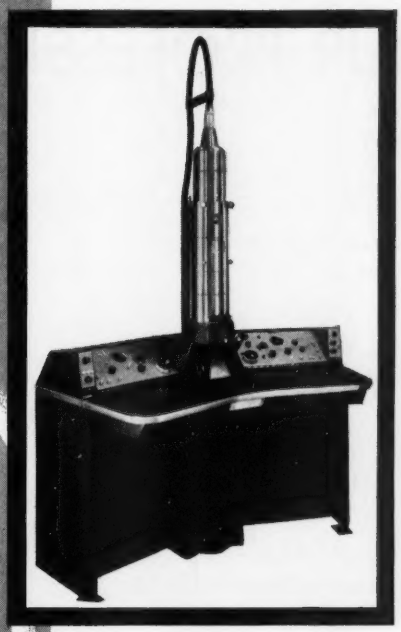

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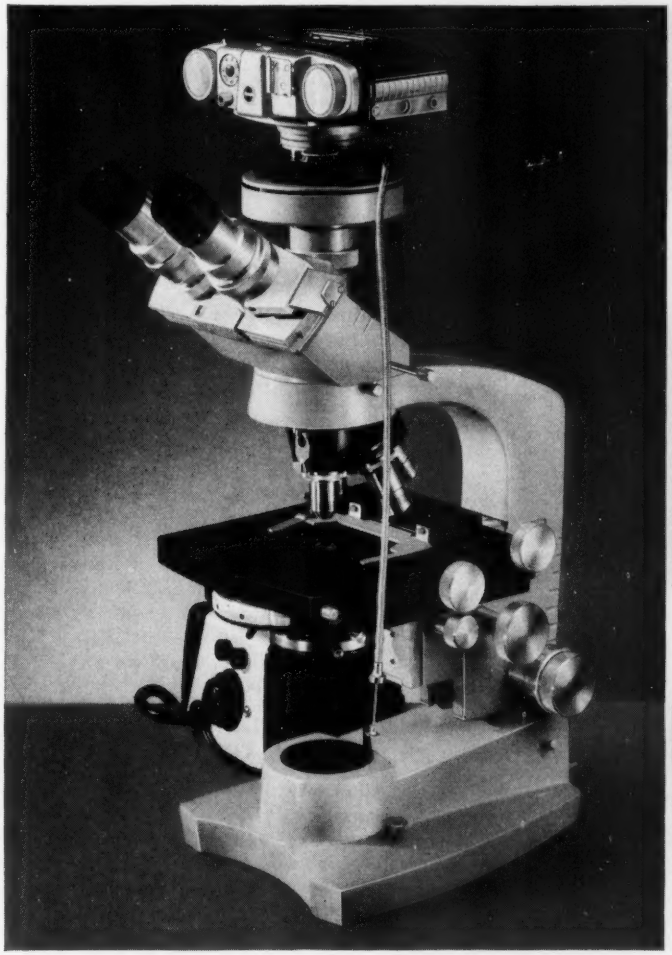

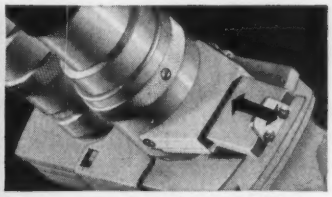

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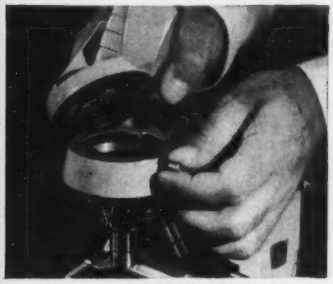

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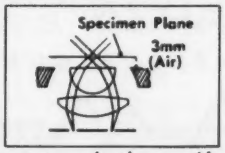

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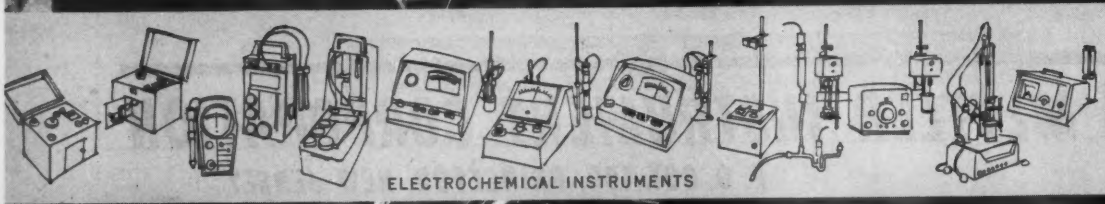
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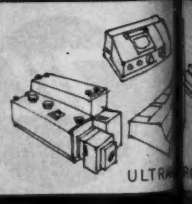
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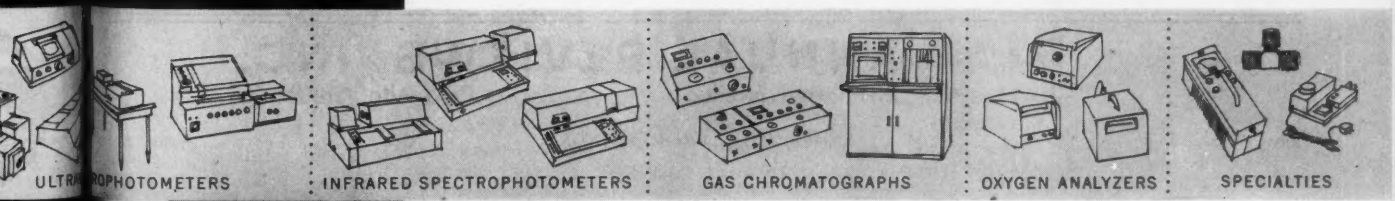

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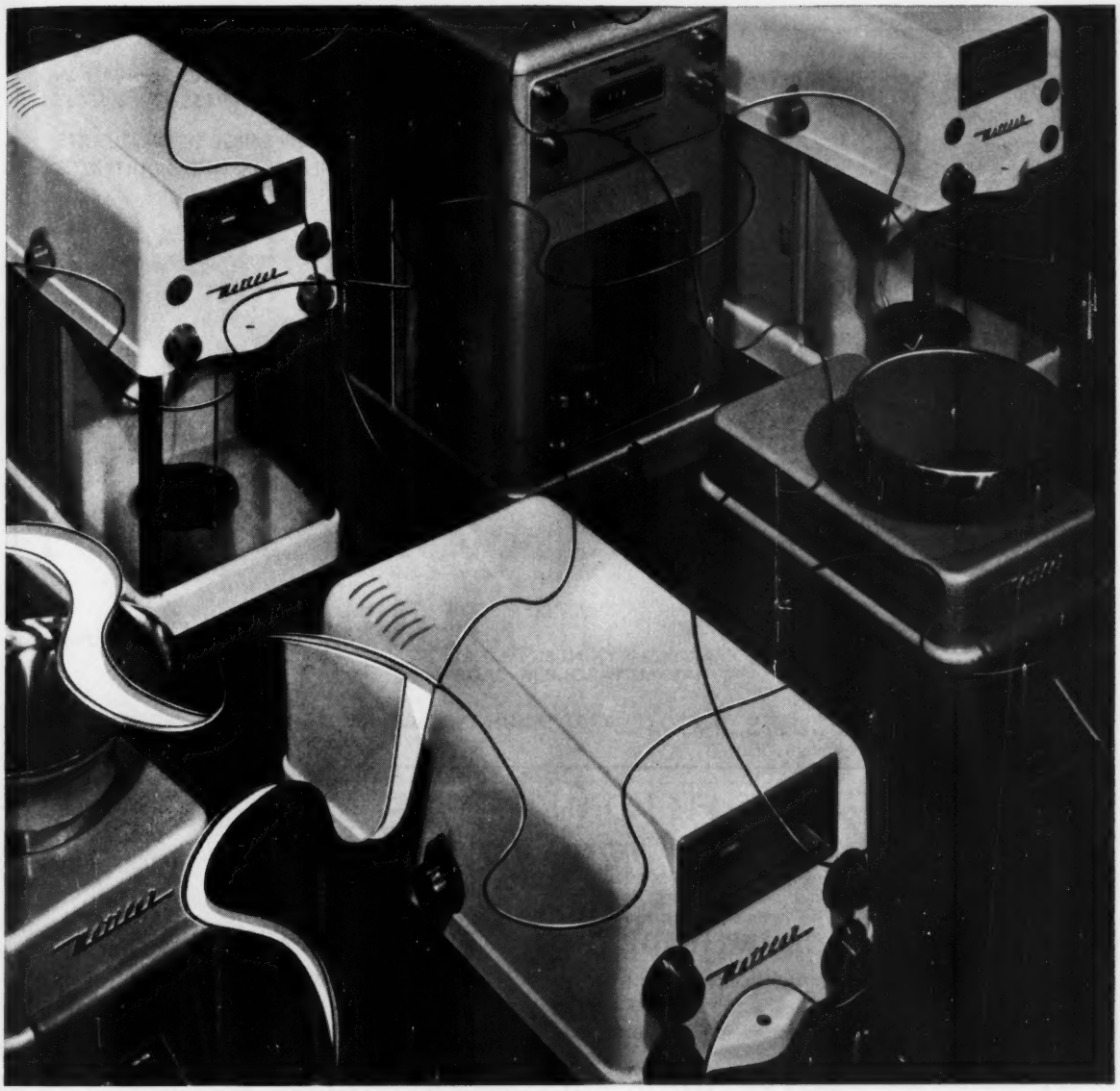

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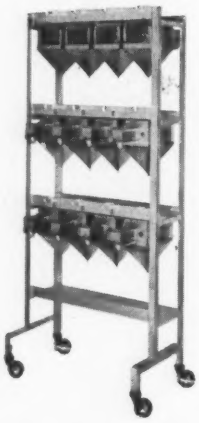

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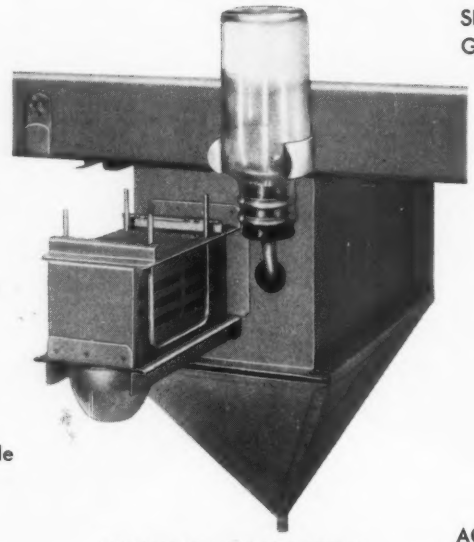

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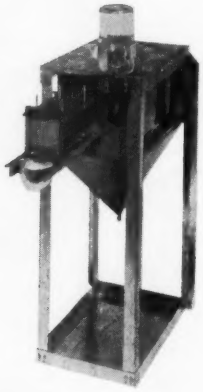

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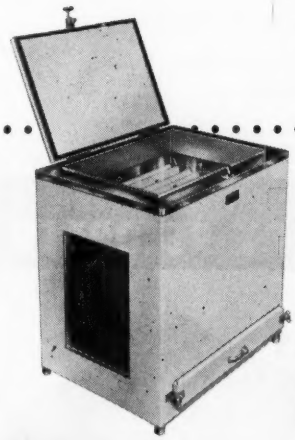

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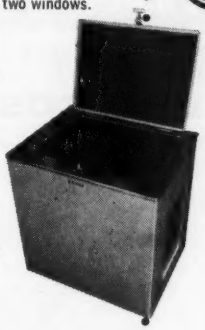

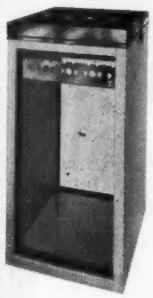

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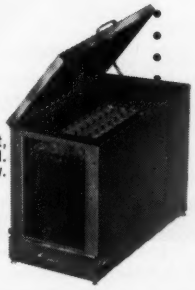

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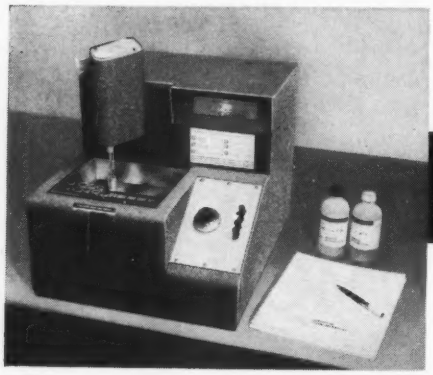

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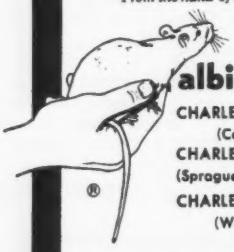

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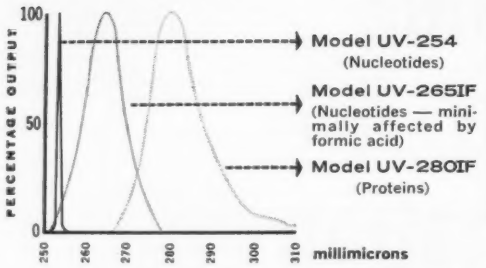

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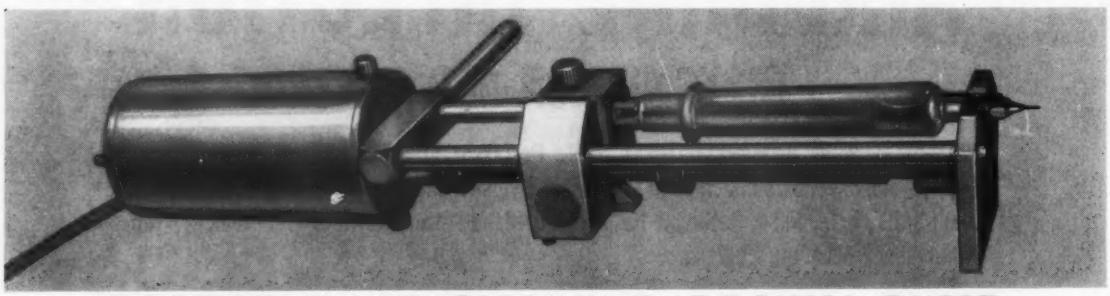

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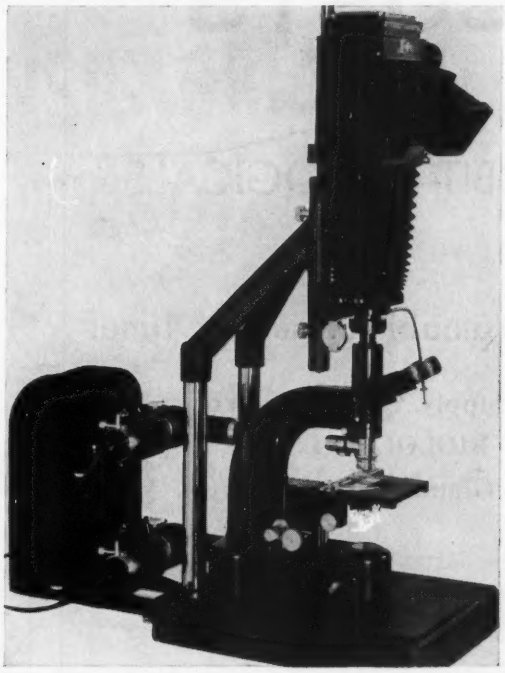

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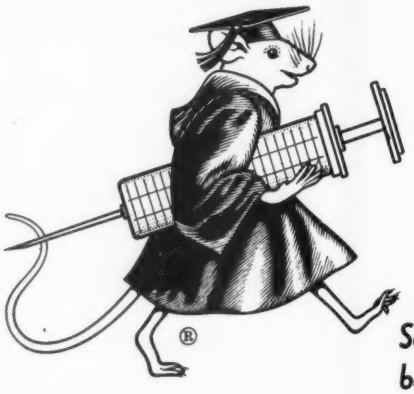

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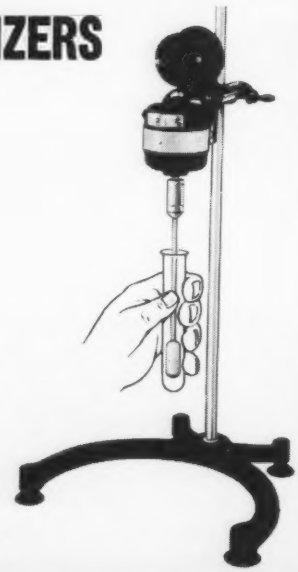

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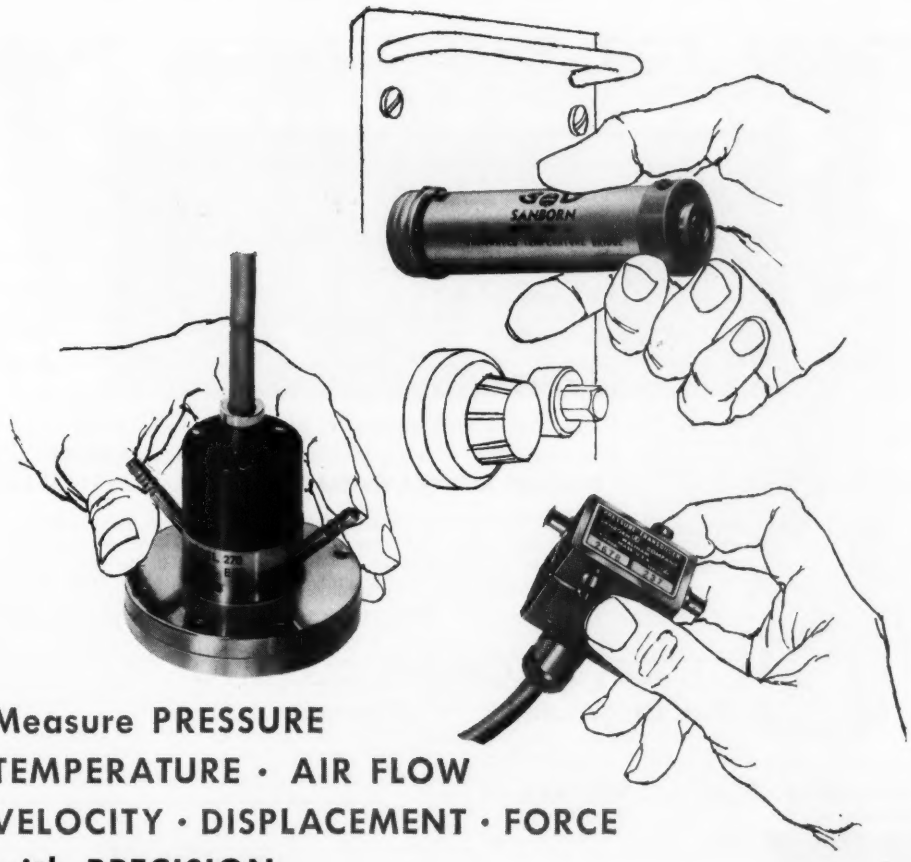

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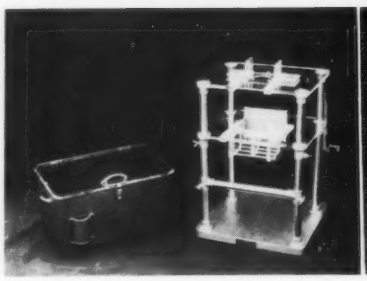

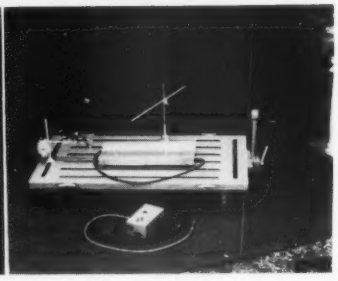

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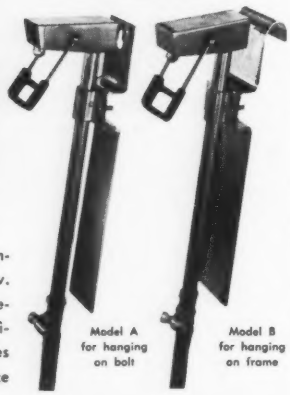

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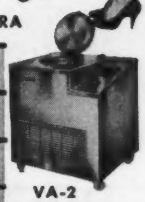
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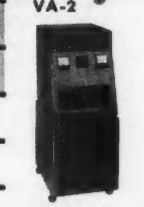
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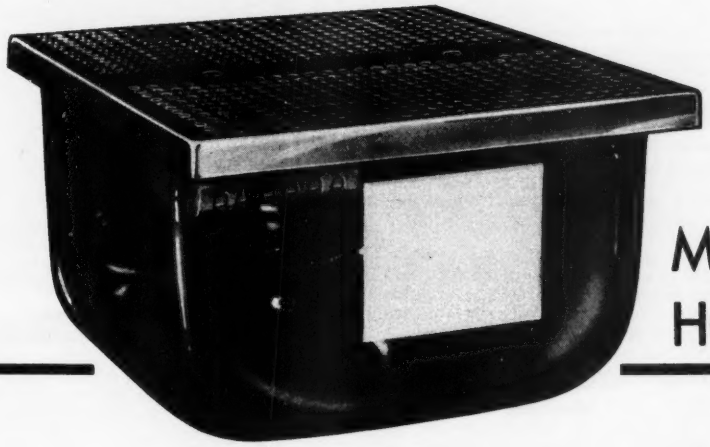

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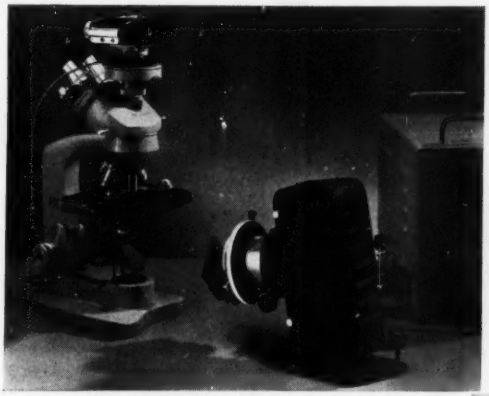

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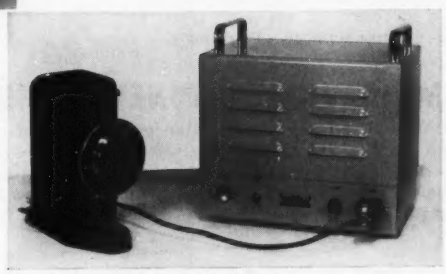

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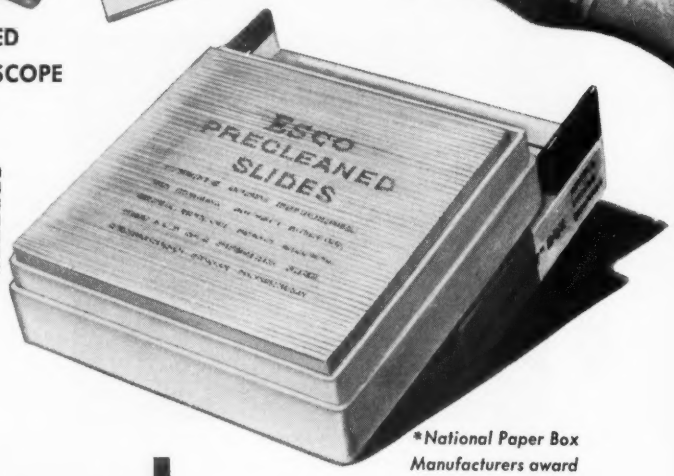

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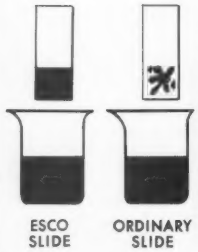

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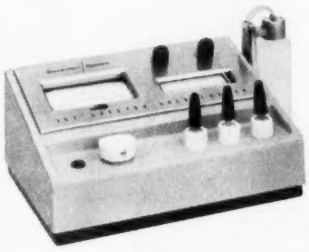

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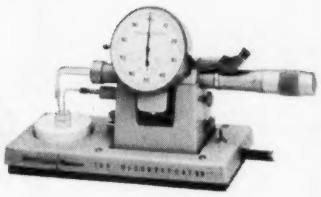

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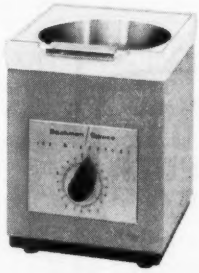

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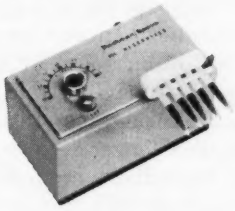

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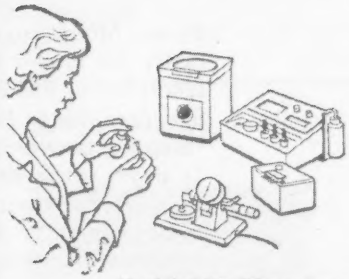

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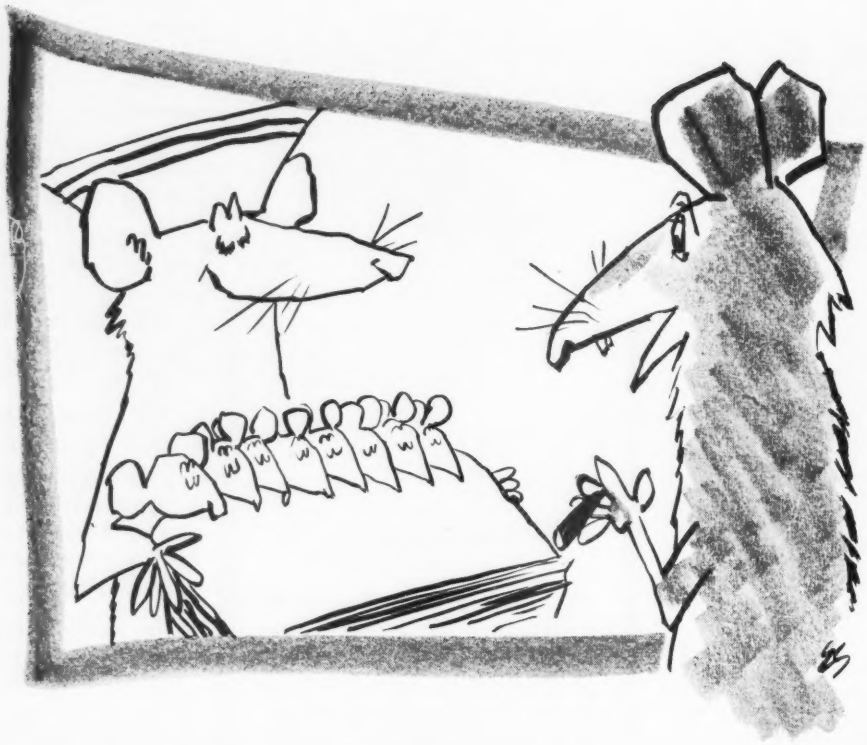

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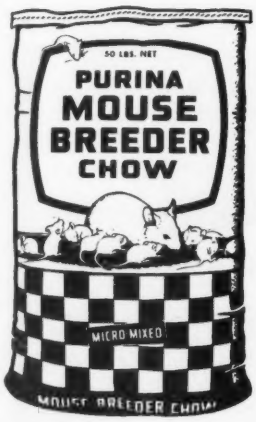

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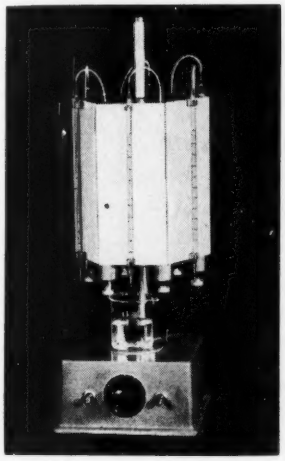

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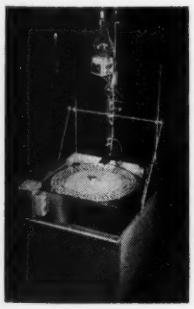

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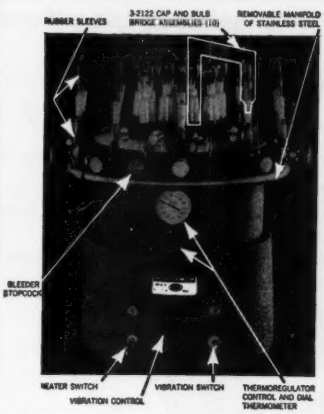

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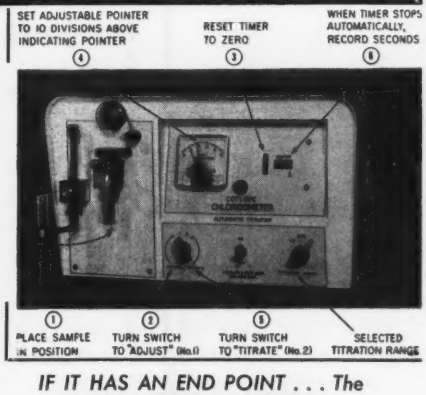

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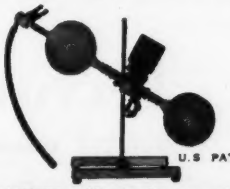

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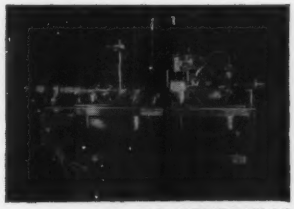

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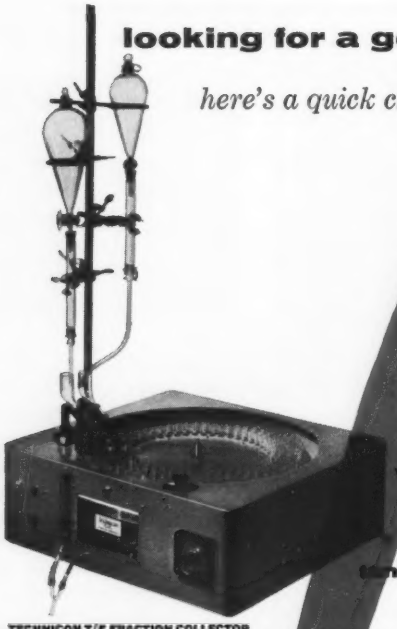

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THE HEMICHOLINIUMS[1]

Introduction

F. W. SCHUELER, *Chairman*
Department of Pharmacology, Tulane University, New Orleans, Louisiana

HEMICHOLINIUMS is a coined term, denoting a group of hemiacetals containing a choline-like moiety. These products are characterized by high toxicity that is slow in onset at minimal lethal doses. The pattern of toxic action reveals interference with cholinergic transmission at such sites as nerve-skeletal muscle junctions, autonomic ganglia, and postganglionic parasympathetic endings.

One hemicholinium in particular, Hemicholinium No. 3 (HC-3 Schueler, 1955), has been rather widely studied by a number of investigators, a few of whom are delivering papers in the present symposium.

Because very considerable work has been done since this subject was last reviewed (actually reviewed in 1958, though printed in 1960—(2)) the contributors to the present symposium have made every effort to develop the subject in an up-to-date way by including not only their personal work, but also that of investigators not participating in the formal presentations.

A number of problems concerning the hemicholiniums in general, and HC-3 in particular, have not been fully resolved, and it is hoped that this symposium may increase our understanding of some of these.

Three outstanding questions are the following: *1*) What are the most sensitive sites (the primary targets) of hemicholinium action? In particular, to what degree is activity presynaptic in locus, postsynaptic, or both, and how are these clearly distinguished? *2*) How are the reported C.N.S. effects of hemicholinium distinguishable from the peripheral effects? *3*) How specific are the chemical structural features for hemicholinium-like action?

Certainly the foregoing, as well as many other or related questions, will come to mind as the symposium progresses.

In closing, the chairman wishes to point out that HC-3 has been found to be a useful tool in the investigation of cholinergic mechanisms, and—as the usefulness of any tool ultimately depends upon the worker's thorough knowledge of its attributes—it is to this end that the present symposium has been designed.

[1] An Intersociety Symposium held in Atlantic City, N.J., April 11, 1961.

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Effect of HC-3 on acetylcholine turnover[1]

F. C. MacINTOSH

Department of Physiology, McGill University, Montreal, Canada

SCHUELER'S PAPER of 1955 (33) identified the hemicholiniums as a group of synthetic choline derivatives which possessed an unusual type of molecular structure and a kind of toxic action that had not been described before. Chemically the hemicholiniums were characterized by the possession of a choline moiety cyclized through hemiacetal formation. Pharmacologically their most striking characteristic was their ability to cause respiratory failure. It was clear that, in exerting this effect, the hemicholiniums were not acting like the familiar neuromuscular blocking agents or like ordinary narcotics. For in the first place, a just lethal dose produced respiratory failure only after a latent period lasting many minutes, or even hours, according to the species; and the length of this latent period was not much shortened by doubling or quadrupling the dose. In the second place, the respiratory failure was not accompanied by generalized neuromuscular block or, indeed, by any other obvious pharmacological effect, unless the dose was far above the LD_{50}. And in the third place, the respiratory depression was antagonized powerfully by eserine and even more strikingly by choline.

These and other observations amply documented Schueler's conclusion that the primary toxic action of the hemicholiniums was directed against some pathway involved in the control of the respiratory muscles. Schueler proposed further, on the basis of evidence that seems now to be of doubtful significance (17, 34), that the locus of respiratory failure was in the spinal cord. Beyond that proposal he did not speculate at length, but he did point out that some features of hemicholinium toxicity recalled the effects of tetanus or botulinus toxin, and he suggested that a cholinergic mechanism was involved.

An obvious possibility, which had occurred to Schueler although he did not state it explicitly in his paper, was that the hemicholiniums were acting as potent and specific inhibitors of acetylcholine (ACh) synthesis. Up to that time no drug with such an action had been identified, although Burgen and his co-workers (7), soon afterward, published a study of the substrate specificity of choline acetylase; in it, they showed that a number of quaternary bases were moderately active in slowing the acetylation of choline by systems containing dissolved choline acetylase and acetyl-coenzyme A (CoA). My colleagues and I at this time were interested in the possibility of inhibiting ACh synthesis pharmacologically, because we were studying the turnover of ACh in sympathetic ganglia and cerebral cortex, and we thought that a specific inhibitor of its synthesis would be a useful tool. We also thought that the pharmacological effects of such a drug might correspond very well to those Schueler had described for the hemicholiniums. A drug whose only action was to stop ACh synthesis would not produce an immediate failure of transmission at cholinergic junctions, for block would only develop when most of the ACh already present in the nerve endings had been used up. Furthermore the length of the latent period before the onset of block would not be very sensitive to the dosage of the drug: it would be mainly determined by other factors, namely the frequency of excitation, the amount of ACh initially present in the nerve endings, the proportion of this ACh released by each arriving impulse, and the initial safety factor for transmission (i.e., the excess of transmitter action above what was necessary to elicit a conducted postsynaptic response). On this basis, the respiratory pathways, because of their incessant activity, would be especially susceptible to paralysis. One would expect choline to be an antidote, because it would promote ACh synthesis in the presence of a competitive inhibitor, whereas eserine would also be an antidote, but because it would increase the effectiveness of ACh released in suboptimal amounts. With these ideas in mind, we were very pleased when Dr. Schueler gave us a generous supply of his hemicholinium compound no. 3, now usually referred to as HC-3.

Our results with the cat's superior cervical ganglion were unequivocal, and, as my co-workers and I have reported them in some detail elsewhere (2, 19–21), I need only summarize them briefly here. You will recall that this small structure is most convenient for studying ACh metabolism: first, because it has a high density of cholinergic synapses, which can be activated at any desired frequency by appropriate stimulation of the

[1] With support from the National Research Council of Canada and the Defence Research Board of Canada.

preganglionic trunk; and, second, because its circulation can be completely isolated for perfusion experiments. After inactivation of the ganglionic cholinesterase, the ACh released by stimulation is quantitatively recovered in the venous effluent and can be measured by bioassay; and, at the end of the experiment, the ganglion can be extracted for assay of the residual ACh. The initial ACh content may be estimated by extracting the unstimulated ganglion of the opposite side, since the two ganglia have practically the same resting ACh content. With reasonable care, all the estimations I have mentioned can be made with an accuracy of about 5%, and, since one can measure the change in ganglionic ACh content as well as the total amount of ACh discharged, one can calculate how much ACh has been synthesized in each experiment.

In the absence of HC-3, a perfused and eserinized ganglion will synthesize ACh even at rest (2), but the rate of synthesis is greatly accelerated by preganglionic stimulation, which releases ACh from the ganglionic depots. If the perfusion fluid is plasma, or Locke's solution supplemented with choline, synthesis in a stimulated ganglion is always adequate to keep up with release, and the ganglion actually increases its content of ACh while the ganglion is being stimulated (Figs. 1 *A* and 2). If the perfusion fluid is choline-free, some synthesis occurs, but not enough to keep up with the output, which declines progressively until it matches the low rate of synthesis; there is a corresponding reduction in the ganglion's content of ACh (Figs. 1 *B* and 2). The addition of HC-3 (2×10^{-5} M) to the perfusing plasma or Locke's solution almost completely deprives the ganglion of its capacity to synthesize ACh: in consequence both ACh output and ACh content decline to a very low level (Figs. 1 *C*, and 1 *D*, and 2). These effects of HC-3 can be prevented or reversed by adding an excess of choline to the perfusion fluid.

HC-3 has no direct effect on the ability of the ganglion to release ACh, as is shown by the fact that the initial output of ACh is the same in its presence as in its absence; neither does it interfere with the action of ACh on the ganglion cells or with conduction in either preganglionic or postganglionic fibers. One would therefore expect that it would have no effect on the transmission of impulses through the ganglion unless the ganglion's store of ACh had been depleted by the arrival of a long series of preganglionic impulses: its action, in other words, would be presynaptic, and secondary to its capacity to inhibit ACh synthesis. Figure 3 shows that this expectation is realized: it is the record of a typical experiment in which the response of the nictitating membrane served to indicate the proportion of impulses reaching the periphery; in this test the ganglion was neither perfused nor eserinized. In the presence of HC-3 the response to postganglionic stimulation at 20 per sec could be maintained indefinitely, but the response to preganglionic stimulation began to fail within 5 min and was, eventually, almost completely extinguished. When conduction in the ganglion had been blocked in this way, it could be partly restored by rest, or by reducing the frequency of stimulation (Fig. 3), or by injecting a dose of choline that, by itself, was too small to affect the ganglion or nictitating membrane. Thus, the effects of HC-3 on transmission through the ganglion corresponded well with its effect on the ACh metabolism of the ganglion. It seems, indeed, that with ganglionic junctions, one can satisfactorily account for

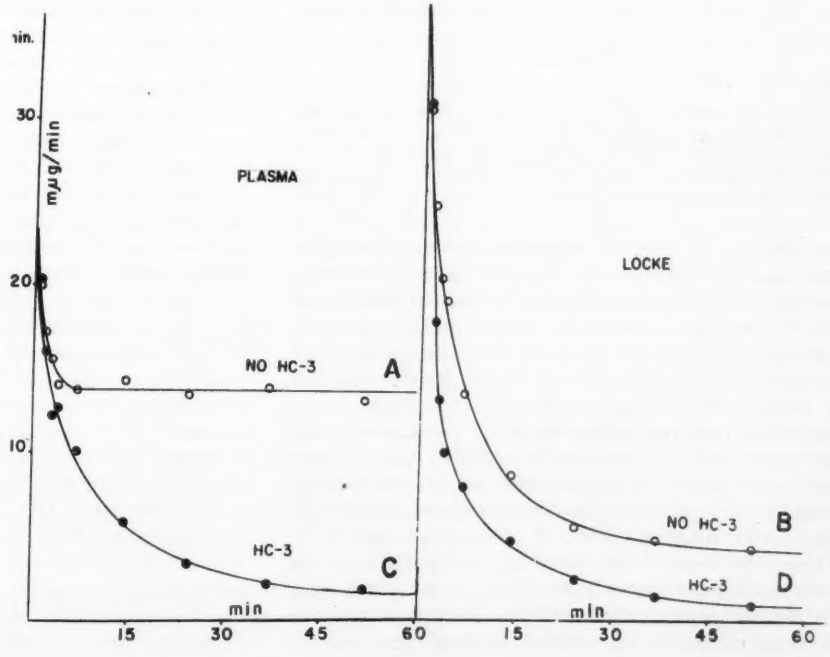

FIG. 1. Rate of ACh discharge from perfused, eserinized ganglia during prolonged preganglionic stimulation (supramaximal, 20/sec, for 1 hr). Each curve is mean from 5 experiments. Perfusion fluids gassed with O_2: lower initial ACh output with plasma perfusion was due to unphysiologically low CO_2 tension; concentration of HC-3, when present: 2×10^{-5} M.

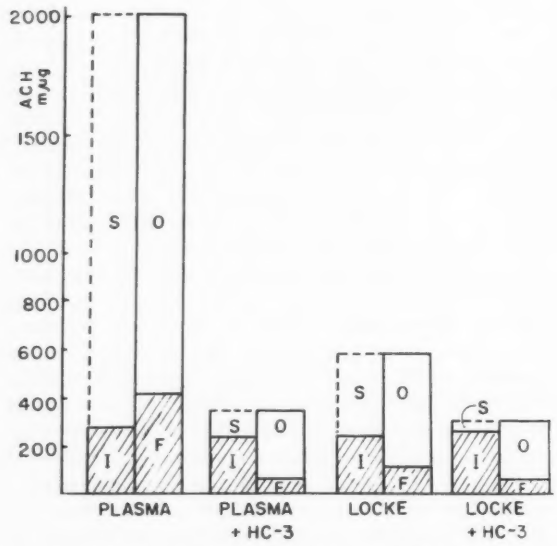

FIG. 2. ACh turnover in perfused, eserinized ganglia. Same experiments as Fig. 1: I = initial ACh content of ganglion; F = final ACh content (after 1 hr's stimulation); O = total output of ACh during stimulation; S $(=O+F-I)$ = ACh synthesized during stimulation.

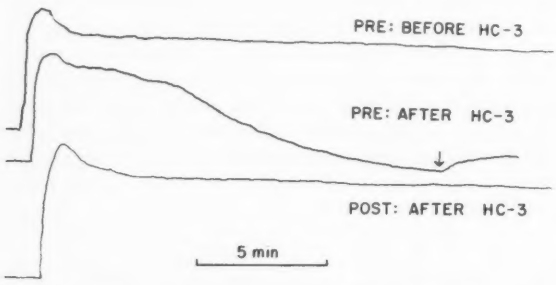

FIG. 3. Response of nictitating membrane of cat to preganglionic and postganglionic stimulation at 20/sec: HC-3, 2 mg/kg, given intravenously before each of 2 lower records; upper record made before administration of HC-3. At *arrow*, in middle record, stimulation frequency reduced to 5/sec.

the effect of the drug on transmission without having to postulate any other mode of action than its observed ability to inhibit ACh synthesis. We have observed that other peripheral autonomic pathways are similarly affected by HC-3: that is to say, they also exhibit junctional block, but only when the traffic of nerve impulses is heavy and prolonged. Schueler's (33) early results suggested that the parasympathetic pupilloconstrictor pathway is affected in this way by HC-3, and the same appears to be true of cardiac vagal (21), chorda tympani secretory (21, 36) and splanchnic-adrenomedullary (36) pathways. Recently B. C. R. Strömblad and P. E. Dresel (personal communication), at the University of Manitoba, have made similar observations with respect to the postganglionic vasodilator fibers supplying the parotid gland: an interesting finding, because this atropine-resistant innervation has been suspected of being noncholinergic. Last, among autonomic junctions affected by HC-3, I ought to mention the remarkable results recently described by Rand and Chang (10, 28). Here the combination of HC-3 and repetitive stimulation was found to lead to delayed block in certain pathways which, by the classical tests, would be regarded as adrenergic, rather than cholinergic. The authors interpret their findings in terms of the hypothesis of Burn and Rand (8, 9), that some fibers that are apparently adrenergic may really be cholinergic, acting by the synaptic release of ACh in proximity to a peripheral cellular store of catecholamine.

We have not systematically studied the action of HC-3 at the neuromuscular junction; but in view of the communications that are to follow I might make brief reference to some recent findings of Dr. S. P. Bhatnagar, in my laboratory. Bhatnagar developed an improved method for estimating ACh in striated muscle: the concentration varies widely from muscle to muscle, but is, of course, always much lower than in ganglia. In cat leg muscles, about two thirds of the stored ACh was lost within a week after ventral root section, and so had presumably been located in the intramuscular nerve twigs and their endings: the rest is probably in the muscle fibers themselves, since unilateral lumbar sympathectomy does not measurably affect the muscle content of ACh. In further experiments Bhatnagar noted that prolonged tetanic stimulation of the motor nerve (maximal shocks at 20 per sec for 30 min) reduced the extractable ACh by about 25%, whereas after the administration of HC-3 the same stimulation resulted in a reduction of 60%. Since, in these last experiments, most of the residual ACh must have been in structures other than nerve endings, it appears likely that the pool of releasable ACh must have been drained nearly dry. In the absence of motor-nerve stimulation HC-3 did not change the muscle's content of ACh. Bhatnagar did not measure ACh output, and his parameters of stimulation were certainly unphysiologically severe; but his results do conform with the idea that HC-3, in some circumstances at least, has a significant presynaptic action at the neuromuscular junctions: for by analogy with what we have seen in the ganglion experiments, one may feel rather confident that, in a muscle depleted of its ACh, each nerve impulse would release an abnormally small quantity of transmitter from the depleted store. I do not mean to suggest that the blocking action of HC-3 at the neuromuscular junction is exclusively, or even mainly, presynaptic. In large doses, it can certainly behave like a conventional postsynaptic blocker, acting rapidly to antagonize the effect of released ACh in much the same way that curare does (29, 33, 34). With smaller doses, on the other hand, there are some findings (22; personal communication from V. B. Brooks and R. E. Thies), that are consistent with a postsynaptic mode of block and others (12, 29, 36) that appear to fit better with a presynaptic mode, and the picture appears to me to be much harder to clarify than it is with the ganglionic

synapses. New experimental results, including those to be presented by other contributors to this symposium, will no doubt help us to a better understanding; and I shall venture, in a moment, to offer a hypothesis that might help to reconcile divergent views. Before doing so, however, I should like to give further consideration to the way in which HC-3 inhibits ACh synthesis.

When we received our first sample of HC-3 from Dr. Schueler, I suggested to my colleague, Dr. P. B. Sastry, that he should test its effect on the synthesis of ACh by minced brain. Quastel and his collaborators (24, 27) showed 25 years ago that brain tissue, respiring in the presence of glucose and eserine, forms ACh in considerable quantity: some of the newly made acetylcholine remains in the tissue as "bound" ACh and some diffuses into the medium as "free" ACh. Working with their system Sastry (32) found that HC-3 strongly inhibited the synthesis of ACh, without, apparently, affecting its partition between the bound and free forms. In the presence of added choline, the inhibition caused by HC-3 was reversed, and ACh was synthesized as fast as, or faster than, usual (Table 1). For nearly complete inhibition of synthesis, a somewhat higher concentration of HC-3 was required than in the ganglion-perfusion experiments (about 10^{-4} M as against 2×10^{-5} M), but this difference could be explained by the presence of significant amounts of free choline in the suspending medium. It seemed reasonable to conclude that, in this system, HC-3 was acting on cholinergic structures in the brain as it did on the ganglionic nerve endings. We assumed that, in either case, its inhibitory action was exerted on choline acetylase, the highly specific enzyme that synthesizes ACh by transferring acetyl groups from coenzyme A to choline. The chemical similarity between HC-3 and choline made it easy to think that the two bases might compete for attachment to the enzyme centers.

To verify this idea Sastry prepared reaction mixtures containing choline acetylase in solution along with acetyl-CoA and choline. The choline acetylase preparation was obtained in the usual way from acetone powders of brain. The mixture synthesized ACh briskly; and again the synthesis was inhibited by HC-3, except when choline was present in excess. In this case, however, HC-3 seemed to be much less potent than we had found

TABLE 1. *Effect of HC-3 on ACh Synthesis by Minced Brain Suspension*

HC-3	Choline	Synthesis*	Free : Total*
M	*M*	μg/g	
		10.3	0.60
	1.4×10^{-5}	9.9	0.51
	10^{-3}	14.2	0.61
10^{-3}		−0.1	0.44
10^{-4}		2.3	0.63
10^{-4}	1.4×10^{-5}	4.2	0.50
10^{-4}	10^{-3}	13.8	0.60

* Synthesis given as micrograms of ACh formed per gram of wet brain during 2 hr at 37 C. Last column gives ratio of free-to-total ACh at end of incubation.

it to be in minced brain or in perfused ganglia: to achieve a 50% inhibition of synthesis in the presence of 10^{-5} M choline, for example, the concentration of HC-3 had to be 10^{-2} M (20, 32). The discrepancy between this weak action of HC-3 on the single-phased system, and its strong action on ganglia, minced brain, and the whole animal, seemed all the more disturbing since choline acetylase, in its natural environment, never has to work at its maximal rate; one can, in fact, calculate that, even if it were to work at 1% of that rate, it could keep up with physiological demands. Moreover, some of the quaternary bases tested by Burgen and his colleagues (7) were more potent than HC-3 in inhibiting the soluble enzyme, although they could not match its pharmacological activity.

We did not feel entitled to make the gratuitous assumption that choline acetylase, in its natural location, was somehow more sensitive to inhibition by HC-3 than after it had been extracted. We concluded, therefore, that HC-3 probably does not act by competing with choline for attachment to the enzyme, and we sought for a different mode of action. We eliminated the hypothesis that HC-3 might act by preventing the formation of acetyl-CoA: the idea was not attractive in view of this ester's key position at the intersection of several important biochemical pathways; moreover, Sastry (32) showed that HC-3 did not interfere at all with the acetylation of sulfanilamide by liver, a classical example of a CoA-dependent mechanism. At this point Dr. Birks made the suggestion that HC-3 might act by combining with some membrane carrier necessary for the transport of choline from the extracellular fluid to the intracellular sites where it is acetylated. The same idea was put forward by Gardiner (14), who had also been impressed by the weaker action of HC-3 on the extracted enzyme than on intact neurons or cellular fragments. Support for the carrier blockade theory came from an experiment suggested by Dr. Arnold Burgen, who pointed out that choline was known (30) to be actively excreted by the avian renal tubule in the same way as certain other quaternary bases such as *N*-methylnicotinamide. The renal experiments were carried out by Birks, and gave a clear result: HC-3 almost completely blocked the excretion of choline, but had no effect on the tubular handling of simultaneously administered phenol red, which is excreted by a different mechanism. The finding by A. Farah—whose personal communication Schueler (37) has referred to—that HC-3 blocks the uptake of both choline and *N*-methylnicotinamide by kidney slices, lends further support to the idea that HC-3 can inhibit the transport of choline, and no doubt of other quaternaries also. Since there was no reason at all to suppose that the renal excretion of choline involves its acetylation, we recorded our opinion (21): that HC-3 (and, by analogy, any other hemicholinium) acts by specifically blocking choline transport across some membrane barrier—the presynaptic membrane itself, or the plasma membranes of surrounding glial cells, or some kind of intracellular membrane—

thereby depriving the choline acetylase within the nerve endings of its raw material. We also noted a number of other facts that appear to fit this scheme for the mode of action of HC-3 presynaptic structures (2, 21).

The hypothesis that HC-3 affects ACh turnover by creating a barrier to the movement of choline is still rather an attractive one. But it remains a hypothesis; and I should like to point out now that a different, though related, hypothesis is equally tenable, and may even fit better with some of the facts that have been discovered by other contributors to this symposium. To introduce this idea, let me remind you that the specific transport, active or passive, of a cation is most readily pictured in terms of some specific anion which has, at least temporarily, a high affinity for the cation, and which is unable to diffuse freely. This anion might be structurally bound to a membrane, but free to rotate as a passive carrier: it would then behave as if it were a specific solvent for the cation, allowing it to diffuse down the electrochemical gradient into the adjacent aqueous phase. Alternatively, the anion might be fixed upon or within some intracellular structure and, by trapping the cation, would permit it to accumulate in high concentration at that site. (More complex models are, of course, possible.) The first alternative, that of the membrane-bound carrier, corresponds with the hypothesis for choline transport that I have just discussed; the second, that of the particle-bound anionic storehouse, is the one I should like to consider now. The well-proven quantal release of acetylcholine at the neuromuscular junction—the discharge of discrete packets containing a thousand or so ACh ions—suggests, as you know, that the acetylcholine of the motor terminal is stored in specific small particles; and, at present, the leading candidates for recognition as the storage organelles are the vesicles that abound within the presynaptic axoplasm. It seems probable that some indiffusible anion may assist in holding ACh within these particles. By analogy, there is strong evidence for the ionic binding of amines to intracellular particles by ATP—serotonin in blood platelets (1, 4) and catecholamines in the adrenal medulla (3); and heparin may similarly aid in the storage of histamine within mast-cell granules (16, 18, 23, 35). If ACh is, indeed, stored in this way within nerve endings, it would not be too surprising if other quaternaries of related structure also had a strong affinity for the specific anionic sites and could be stored there, too. Let us make the further, not unreasonable, assumption that choline and related bases enter the nerve ending mainly at the time of its invasion by the nerve impulse: this could be compared to the well-known influx of Na and Ca ions into excited axons, but it is possible that a pinocytotic process may be involved in the reformation of the transmitter depots. Now, if HC-3 were to be handled by the nerve ending in the same general way as the choline and ACh to which it is sterically related, it would tend to accumulate in the storage sites, and the latter would then contain less than the normal complement of ACh. The transmitter quantum, instead of being pure ACh, would be adulterated with a certain proportion of HC-3, and this proportion would rise with each successive impulse arriving at the junction. The number of transmitter quanta per impulse would be unchanged, but the later impulses of a series would have less effect on the postsynaptic membrane because they would be discharging less ACh and more of the competitive blocker HC-3.

As a result of the usurpation of storage sites by HC-3, net synthesis of ACh during stimulation would be inhibited. The degree of inhibition would depend on the relative extracellular concentration of HC-3 and choline, and on the relative affinity of HC-3 and ACh for the storage sites.

This hypothesis is perhaps overlabored, but I find it attractive, because it would help to account for some of the observations made on neuromuscular junctions poisoned with HC-3 that are hard to explain on the basis of either a pure postsynaptic action or a pure presynaptic action. To take one example, Brooks and Thies (personal communication) have found that the postsynaptic response of an isolated muscle to motor-nerve volleys at 12 per min continues to decay along a smooth curve for more than an hour after the muscle has been placed into contact with HC-3. It is hard to see why a purely postsynaptic effect should take so long to develop. Yet, a purely presynaptic action on the part of HC-3 would be an equally unsatisfactory explanation, for if the proportion of depot ACh released by each nerve impulse were the same for the muscles used by Brooks and Thies as Emmelin and I (13) have found for the cat's tibialis anticus, the ACh content of the motor terminals in their tests should have been reduced by only some 2–3%, at a time when the postsynaptic response was reduced by 50–90%. On the other hand, a dual effect of the sort I have envisaged—the release of transmitter quanta containing a steadily increasing proportion of the postsynaptic blocker HC-3—might well account for these and other findings. Birks and I (2) have recently presented evidence that the transmitter store at ganglionic nerve endings exists as two fractions, one of which is smaller and more readily releasable than the other. It seems probable that at physiological rates of excitation ACh turnover occurs mainly in this smaller fraction, which might then be particularly susceptible to adulteration when another quaternary is present in the extracellular fluid.

We have no direct evidence that HC-3, or any other quaternary besides choline, can be incorporated into nerve endings. We do find, however, that a number of other quaternaries can inhibit ACh synthesis by minced brain. Table 2 contains some of Dr. Bhatnagar's unpublished data on this point. It will be noted that the parent base, NH_4^+, is inactive as an inhibitor, as is *N*-methylnicotinamide; but some of the others, especially tetraethylammonium (TEA), inhibit strongly when present in high concentration: by this test the potency of TEA is roughly 1/20 that of HC-3. Probably it does not act on ACh metabolism in exactly the same way;

TABLE 2. *Inhibition by Quaternary Bases of ACh Synthesis in Minced Brain*

Base	ACh Synthesis as % of Control		
	10^{-4} M	10^{-3} M	10^{-2} M
Ammonium	104	105	103
N-methylnicotinamide	105	103	97
Tolazoline	98	89	84
Hexamethonium	77	73	63
d-Tubocurarine	79	66	53
Tetramethylammonium	83	81	12
Tetraethylammonium	73	32	9
Tetrapropylammonium	80	65	12
Tetrabutylammonium	78	57	6
HC-3	23	14	5

TABLE 3. *Reversal by Choline of Inhibition of ACh Synthesis in Minced Brain*

Inhibitor	ACh Synthesis as % of Control			
	No Choline	Choline 10^{-4} M	Choline 10^{-3} M	Choline 10^{-2} M
None	100	142	176	177
HC-3 (10^{-4} M)	24	62	123	
TEA (10^{-3} M)	37	56	70	144
TEA (10^{-2} M)	17	18	35	70

TABLE 4. *Antagonism by Choline and Eserine to TEA Toxicity in Mice*

Dose of TEA	Antagonist	Dose of Antagonist	Mortality	
			Numbers	Percentage
mg/kg		*mg/kg*		
75			10/20	50
100			70/80	88
150			39/40	98
100	Choline chloride	100	2/20	10
150	Choline chloride	200	4/10	40
100	Eserine sulfate	0.5	4/30	13

nevertheless, it was of interest to us to find that it has no effect on ACh synthesis by choline acetylase in solution, that its inhibitory action on synthesis by minced brain is reversed by choline (Table 3), and also that choline is a fairly effective antidote to the toxic action of TEA in mice (Table 4). Curiously enough, Bhatnagar found that even *d*-tubocurarine and hexamethonium, which we have all regarded as typical drugs with a purely postsynaptic action, have a measurable inhibitory effect on ACh synthesis. It is, indeed, likely that any influence these two bases may have on ACh release is wholly negligible, in ordinary circumstances. Nevertheless, it should perhaps be pointed out that, in each of the perfusion experiments (6, 11, 25) in which these blocking agents were found not to affect ACh release, the presynaptic fibers were excited for only a few minutes in the presence of the drug; and it is just possible that more prolonged stimulation would have given evidence of depletion of the transmitter depot. At present, eserine is the only organic base other than HC-3 that has been shown, by direct measurement, to depress ACh release from nerve endings (2, 26). I feel rather confident, however, that other compounds will prove to possess this sort of activity. Among them may well be triethylcholine and 3,6-bis(diethylaminopropoxy)pyridazine bismethiodide, whose pharmacological similarities to the hemicholiniums have been pointed out by Bowman and Rand (5) and Gesler et al. (15), respectively.

It is a truism that drugs whose activity is specially directed toward cholinergic synapses can act in a variety of possible ways. On the presynaptic side, they may affect the transport or acetylation of choline as well as the storage and release of ACh; on the postsynaptic side, they may mimic or block the action of released ACh; on either side of the junction they may affect the enzymatic or nonenzymatic removal of ACh from its site of action. With exceptions, such as botulinus toxin and the organic phosphates, most of the drugs that have a special action at cholinergic synapses are bases with some steric resemblance to choline or ACh; we should expect, therefore, that the elements with which they tend to combine —receptors, enzymes, carriers, or storage sites—will often possess a common but specific antipodal configuration. Thus it is not surprising that many drugs should act in more than one way at these synapses. When we begin to learn the chemistry of the materials with which choline and ACh specifically combine, we shall be on our way to an understanding of cholinergic pharmacology.

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Postsynaptic neuromuscular block produced by hemicholinium no. 3[1]

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AT PRESENT NO DRUG IS KNOWN that specifically inhibits the synthesis of acetylcholine (ACh) in nerve-muscle preparations. When MacIntosh, Birks, and Sastry (41, 42) reported that hemicholinium no. 3 (HC–3) inhibits ACh synthesis in perfused active sympathetic ganglia and in minced brain (41, 42), the present study was undertaken in order to test its mode of action on guinea pig nerve-muscle preparations. HC–3 is a quaternary base, containing two choline moities (51). The effects of this drug on various tissues and the evidence for its possible modes of action have been reviewed recently by Schueler (52). In this study it will be shown that HC–3 acts postsynaptically under the conditions of our experiments, in contrast to a suggestion of presynaptic action in cat's muscle (20), but in agreement with findings for frog's muscle (44, 45). The comparative neuropharmacology of HC–3 will be discussed.

METHODS

Toxicity

HC–3 toxicity was determined by intraperitoneal injection of water solutions into groups of three or more guinea pigs. Volumes of 0.2–1.8 ml were injected into male or female animals with body weights of 200–500 g. The development of symptoms and the time it took for death to occur were noted. Possible antagonism by choline or by eserine was tested by separate injections of either of these drugs immediately before injections of HC–3 solution (Table 1). Some tests were performed with animals that had survived an experiment done during the previous week.

Preparation and Solutions

For each in vitro experiment, a single, excised, guinea pig muscle was used. The diaphragm was used for one preliminary trial (Fig. 1), whereas 15 serratus nerve-muscle preparations were employed in subsequent studies (4). The experiments are identified by numbers, in the text, to permit cross-referencing between trials. The muscles were mounted in a suitable Lucite bath (6), containing 6 ml of modified Krebs' solution (18). The solution had the following composition (mM): Na^+—143.4, K^+—5.6, Ca^{2+}—1.8, Mg^{2+}—1.0, HCO_3^-—23.8, Cl^-—130.8, and glucose—5.5. In four of the five experiments, during which ACh release was studied, the concentration of Ca ions was raised to 7.2 mM and that of Mg ions to 5.0 mM, except 10 mM Mg for *experiment 12* (Tables 3 and 4). Provision was made for recording from muscles and nerves. The latter were mounted in a separate compartment, filled with mineral oil, and connected to the muscle bath by a slot filled with petrolatum. The modified Krebs' solution was usually warmed to 40 C, equilibrated with 5 % CO_2 and 95 % O_2 in 2-liter reservoirs; it was then run through the muscle bath at a rate of about 5 ml/min, with the bath temperature maintained at 36 ± 1 C. ACh release was studied at lower temperatures, as shown in Table 4. All drugs were added to the reservoir containing the Krebs' solution.

Electrical Recording

Extracellular muscle-action potentials and end-plate potentials (epp's), in response to nerve stimulation, were recorded from the surfaces of six muscles (*expts. 1, 2, 5, 6, 10,* and *11*) with glass-insulated platinum wires, 0.3 mm in diameter (5). Intracellular (full and miniature) end-plate potentials were recorded from single fibers in *experiments 2* and *3*, from three fibers in *experiment 9*, and, in *experiments 12–16* (Tables 3 and 4), with glass micropipettes, filled with 3M KCl solution. Potentials were amplified with a d-c amplifier, displayed on an oscilloscope and photographed.

Quantum Content

Quantum content was estimated by analysis of the coefficient of variation of series of epp amplitudes (13), corrected for nonlinear summation (43). Series of about 200 epp amplitudes were used for the calculations, resulting in mean estimates of quantum content with standard errors approaching 10 %. During recovery

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in trials 2 and 3, *experiment 13* (Table 3), the epp amplitude increased gradually. Consequently, quantum content was determined by deviations from the linear regression of epp amplitudes, rather than from mean epp amplitude.

ACh Sensitivity

Postsynaptic sensitivity to ACh was tested by application of 0.01–0.05 ml of solution of ACh in 0.9% NaCl to end-plate regions of five muscles (*exbts. 4–8*, Table 2) through a 25-gauge needle from a 0.25 ml syringe that was held in a mechanical manipulator. The tip of the needle was placed in the irrigating solution 0.3–0.4 mm from the muscle. Muscle twitches were observed through a dissecting microscope at a magnification of 16–25 diameters. The final concentration of ACh reaching the postsynaptic surface of muscle cells was unknown.

Drugs

HC–3 was obtained through the courtesy of Dr. K. K. Chen of the Lilly Research Laboratories, Indianapolis. In some experiments, choline chloride (chemically pure) was included in the Krebs' solution at a concentration of 1 or 2 mM, as a possible antagonist to the action of HC–3. Acetylcholine chloride, obtained from Merck & Company of Rahway, N. J., was used to test the postsynaptic sensitivity of neuromuscular junctions. The contents of dry, sterile ampules were dissolved in a 0.9% NaCl solution and successively diluted to produce a series of solutions with concentrations of 10^{-1}–10^{-6} M. Eserine sulfate (Physostigmine, U.S.P., Amend Drug & Chemical Co., New York City) was dissolved in a 0.9% NaCl solution to produce a concentration of 10^{-5} g/ml.

RESULTS

1. Toxicity of HC–3 for Guinea Pigs

The toxicity of HC–3 has been demonstrated for a variety of mammals (32, 49, 51). Since Schueler's (51) extensive tests included few guinea pigs (the species investigated in this study), HC–3 toxicity and its possible antagonism by other drugs were reexamined (Table 1). Fractions appearing in Table 1 represent the number of animals surviving relative to those tested with the combinations of drug doses shown at the top and in the first column. The vertical columns in Table 1 list trials with constant doses of HC–3, whereas the rows itemize constant doses of antagonistic drugs used. The approximate LD_{50} for guinea pigs was 0.5 mg of HC–3 per kg body weight, as shown in Table 1. HC–3 caused extreme slowing of respiration, flaccid paralysis, and convulsions, as described for other mammals (32, 49, 51). Death was apparently caused by respiratory failure, usually in 30–60 min. Choline and eserine were tested as possible antagonists to HC–3 toxicity with guinea pigs, since these drugs were effective with mice (51). On the one hand, only slightly greater doses of choline (100 mg/kg) than those used by Schueler (51) for mice were required to antagonize otherwise lethal doses of HC–3 in guinea pigs (Table 1), although the LD_{50} of HC–3 expressed per unit of body weight for guinea pigs is 20 times that for mice (51). On the other hand, eserine was ineffective; maximal sublethal doses of eserine (1 mg/kg body wt) failed to improve the chances of survival, at least with the small numbers of animals tested (Table 1).

TABLE 1. *Toxicity of HC–3 to Guinea Pigs and Its Antagonism by Choline (but not by Eserine)*

Doses (mg/kg body wt)		Controls	HC-3 (mg/kg)					
			0.3	0.5	1.0	2.5	5.0	10.0
		3/3 (placebo)	3/3	4/10 (62 min)	0/5 (41 min)	0/3 (41 min)	0/3 (32 min)	0/3 (21 min)
Eserine (mg/kg)	1	4/4		3/6 (42 min)	0/3 (34 min)			
	2	1/3 (28 min)		0/3 (28 min)				
	2.5	2/3 (10 min)						
Choline (mg/kg)	20				3/3*			
	50				2/3 (47 min)			
	100				3/3			
	250	3/3				1/3 (54 min)		
	500	1/3 (76 min)				1/3 (16 min)		

The number of animals "surviving" is shown as a fraction of the number of animals tested, for the combination of drugs listed at the top and left side. Figures in parentheses are average times for death. Drugs were injected intraperitoneally at zero time into animals of both sexes (body wt 200–500 g) as volumes of 0.2–1.8 ml.

* Two of the three animals had severe symptoms of HC-3 poisoning.

2. General Site of Action: Neuromuscular Junction

It is known that frequencies of indirect stimulation greater than 12/min fatigue isolated mammalian nerve muscle preparations (11 and unpublished data). Therefore, to avoid fatigue, the nerves of excised diaphragm or serratus preparations were stimulated supramaximally at frequencies of 12/min or less. HC–3, in concentrations as low as 3×10^{-5} M, blocked neuromuscular transmission reversibly at some junctions. Transmission at all junctions failed in the presence of 2×10^{-4} M HC–3, after 30–40 min with a diaphragm preparation, and after 10–20 min with serratus preparations (Fig. 1). Doses of HC-3 that cause considerable neuromuscular block in vitro (6×10^{-5} M, about 3 mg/100 ml) exceed by more than 10-fold the expected concentrations of HC-3 in the blood of severely affected guinea pigs (0.5 mg/kg), even if only half of the injected dose can be assumed to reach nerve-muscle junctions (52). The five curves of Fig. 1 show rates of depression of compound muscle action potentials recorded with surface electrodes at end-plate regions of different muscles. Amplitudes of end-plate potentials (epp's) are designated by filled symbols in lower part of Fig. 1. The presence of epp's in the absence of propagated action potentials demonstrates that ACh was still released from nerve endings in response to nerve stimulation. Control values for nerve potentials and for muscle potentials elicited by direct stimulation (top of Fig. 1) demonstrate that HC–3 acts at neuromuscular junctions rather than on nerves or on muscle fibers. Neuromuscular block was partially antagonized by addition of 4×10^{-5} M eserine to irrigating solution containing 9×10^{-5} M HC–3 (*expt. 11*, not shown in Fig. 1). Recovery from block due to HC–3 (not shown in Fig. 1) was observed during irrigation of the preparation with drug-free Krebs' solution: the time course of recovery was variable but similar to that of development of paralysis.

3. Specific Site of Action: Postsynaptic Muscle Surface

Two types of experiments were made to distinguish between presynaptic and postsynaptic effects of HC–3 on the neuromuscular junction: muscle sensitivity to applied ACh was determined by observing twitch threshold, and electrical records were obtained from single junctions. The results are described below.

a) Depression of sensitivity to externally applied ACh. Normal ACh sensitivity is contrasted with that observed during irrigation with modified Krebs' solution containing HC–3 (Table 2). Application of ACh to end-plates of groups of normal serratus muscle fibers caused them to twitch, in response to concentrations of about 10^{-5} M ACh, as has been previously found (7, 8). Within an hour after addition of blocking doses of HC–3, threshold concentrations of ACh required to produce twitches increased about 100 times. Sensitivity was partially restored by irrigating with drug-free Krebs' solution for 20–90 min (Table 2). Recovery of sensitivity was followed

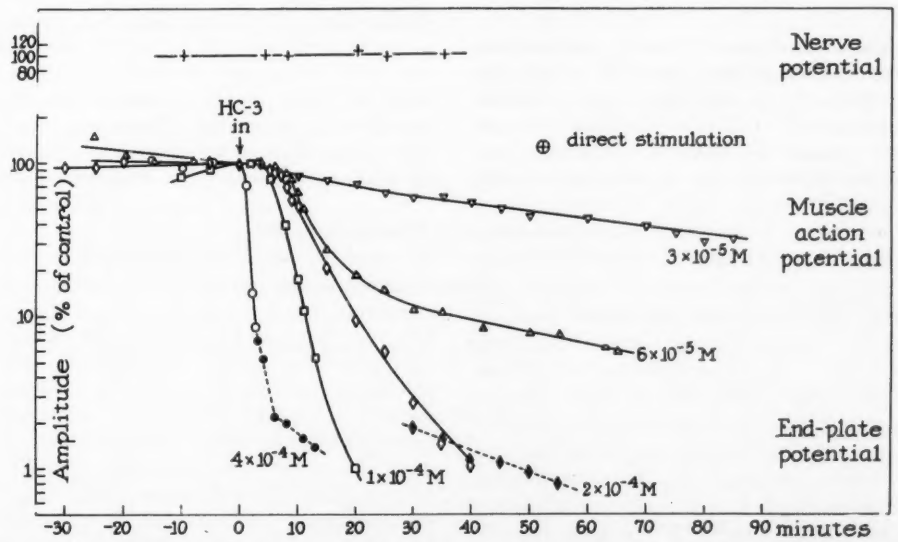

FIG. 1. Time course of neuromuscular block with various concentrations of HC-3. Five guinea pigs' excised nerve-muscle preparations were stimulated indirectly and supramaximally at 12/min. HC-3 reached the bath from the reservoir at zero time. The uppermost horizontal line plots amplitude of control "nerve potentials," expressed as percentage of normal (ordinate, log scale), against time (min). +: phrenic nerve (*Expt. 1*), recorded in oil-filled side chamber. Open symbols plot amplitudes of "muscle action potentials" (ordinate, log scale) in relation to time (abscissa). ◇: diaphragm (*Expt. 1*), 2.3×10^{-4} M HC-3, ⊕: direct stimulation; ▽: serratus (*Expt. 5*), 3×10^{-5} M HC-3; △: serratus (*Expt. 6*), 6×10^{-5} M HC-3; □: serratus (*Expt. 10*), 1.2×10^{-4} M HC-3; ○: serratus (*Expt. 2*), 3.7×10^{-4} M HC-3. Filled symbols represent amplitudes of "end-plate potentials" expressed as percentage of control muscle action potentials. ◆: diaphragm (*Expt. 1*), 2.3×10^{-4} M HC-3 and ●: serratus (*Expt. 2*), 3.7×10^{-4}. Bath temperatures were 35–36 C, except 25 C. in *Expt. 10* (□).

only until compound muscle action potentials leveled off without further recovery; the residual loss may be attributable to long-lasting depression of sensitivity after repeated application of ACh (54). Thus the extent of depression of ACh sensitivity, about 100×, can roughly account for the depression of muscle action potentials, shown in Fig. 1. The effects of eserine and choline on ACh sensitivity were tested. In the presence of eserine, HC–3 increased the concentration of ACh required to stimulate muscle fibers only 5–20 times instead of 100 times (Table 2). Choline, however, was ineffective in reversing the desensitizing action of HC–3 at neuromuscular junctions, although it antagonizes the toxicity of HC–3 (Table 1, and ref. 51), and counteracts block by HC–3 of transmission at cholinergic autonomic junctions (41). In two tests, addition of 1 mM choline further depressed sensitivity and the compound muscle action potential, rather than restoring them (Table 2), as is also the case with cat's muscle in vivo (28). Rinsing with drug-free Krebs' solution led to partial recovery of ACh sensitivity and of muscle potentials.

b) Depression of amplitude of miniature end-plate potentials. The postulate that HC–3 desensitizes the postsynaptic muscle membrane to applied ACh implies that sensitivity to spontaneously released ACh should be decreased as well. The properties of spontaneous miniature epp's were used to determine the site of action of HC–3. Del Castillo and Katz (15) and others have demonstrated that miniature epp's are postsynaptic responses to spontaneous presynaptic release of single quanta of ACh. Such release can be demonstrated by direct bio-assay of ACh (5, 53).

Toxins, ionically imbalanced irrigating solutions, and mechanical factors that act presynaptically change the frequency of discharge of miniature epp's, whereas drugs and imbalanced irrigating solutions that act postsynaptically change the recorded amplitude (33). Thus, it may be expected that intracellular records from single unstimulated junctions can indicate whether HC–3 acts presynaptically or postsynaptically. A change of quantal size of miniature epp's has never been produced experimentally (tests for such an effect will be discussed in the next section). It was found that doses of HC–3 sufficient for neuromuscular block (6–18 × 10^{-5} M) immediately obliterate spontaneous miniature epp's (three tests in two fibers, *Expts. 2* and *3*), but that lower doses, such as 10^{-5} M HC–3, gradually depress their mean amplitude into the base line "noise" over a period of 5–10 min without changing their time-constant of decay. Yet, resting membrane potentials were not depressed by these treatments with HC–3. Fig. 2 shows distributions of miniature epp amplitudes for a series of recording periods of 13 sec each from the first fiber of *experiment 9* (representative of trials with three fibers, *Expts. 3* and *9*). The numbers of miniature epp's are indicated on the ordinate and amplitudes are grouped along the abscissa in class widths of 0.05 mv. Arrows on the abscissa indicate limits of resolution of recording; miniature epp's with amplitudes of 0.1 mv or less could not be distinguished from base line noise.

The histograms at left on Fig. 2, beneath the label "HC–3," demonstrate depression of miniature epp amplitude by HC–3. Time after addition of HC–3 is designated next to the histograms. One min after HC–3 entered the muscle bath the miniature epp amplitudes were still equivalent to control values; but 2 min after addition of HC–3, amplitudes of miniature epp's had diminished, and then continued to decline with time. Since miniature epp's could not be counted after they disappeared into the base line noise, frequencies appear to be reduced, as reflected in the decreasing areas of successive histograms. However, results of experiments with low doses of HC–3 have shown that frequency reduction is an artifact. For example, in the second fiber of *experiment 9*, miniature epp amplitude decreased by about one quarter after treatment with 3 × 10^{-6} M

TABLE 2. *Depression of ACh Sensitivity by HC–3*

Expt. No.	Concentration of HC-3 in Krebs' Solution (M)	Additional Drugs Used (M)	Threshold Sensitivity to Applied ACh			Duration of Irrigation with Krebs' Solution		
			Control before HC-3 (M)	In presence of HC-3 (M)	Recovery from HC-3 (M)	Control before HC-3 (min)	In presence of HC-3 (min)	Recovery from HC-3 (min)
4	1.2×10^{-4}		3×10^{-5}	$<10^{-3}$	10^{-3}	220	35	25
5	2.7×10^{-5}		3×10^{-5}	10^{-3}	10^{-4}	330	90	90
6	6.2×10^{-5}		3×10^{-5}	10^{-2}–10^{-2}		195	60	
	6.2×10^{-5}	Choline: 10^{-3}	10^{-5}	10^{-2} and less	10^{-4}		85	40
	6.2×10^{-5}	Choline: 10^{-3}	10^{-4}	10^{-2}	10^{-4}		15	20
7	2.3×10^{-4}	Eserine: 3×10^{-5}	2×10^{-5}	10^{-4}		160	70	
8	1.2×10^{-4}	Eserine: 3×10^{-5}	5×10^{-6}	10^{-4}		210	130	

Tests at 3–6 loci in each of five guinea pig excised serratus muscles. Indirect stimulation at 12/min during *expts. 5* and *6* which also appear in Fig. 1; no stimulation during other experiments. Muscle was irrigated with drug-free Krebs' solution between the two tests in the presence of choline.

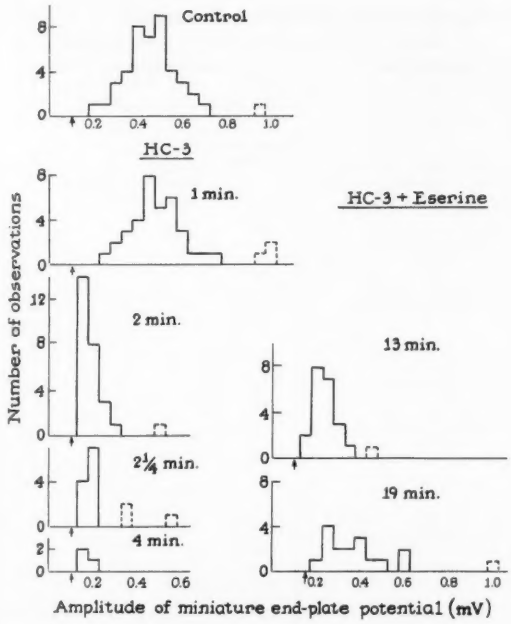

FIG. 2. Changes of distributions of amplitudes of spontaneous miniature epp's. First fiber of guinea pig's excised serratus muscle used in *experiment 9*. Numbers of potentials observed (ordinate) are plotted at various amplitudes (abscissa) in classes of 0.05 mv width. The number next to each histogram indicates minutes after addition of HC-3 (1.2×10^{-6} M); eserine (1.2×10^{-5} M) was added 10½ min after HC-3. Amplitudes of miniature epp's that are double or triple the mean amplitude are shown by an interrupted outline. Limit of resolution imposed by base-line "noise" is shown by arrows on abscissae; miniature epp's with amplitudes of 0.1 mv and below were not detected.

HC–3. Subsequent exposure to 6×10^{-6} M HC–3 further depressed mean amplitude to less than one half of control. Yet, since all amplitudes were above base line noise, it was possible to establish that there was no effect on frequency of miniature epp's.

If HC–3 depresses postsynaptic sensitivity to ACh, then an anticholinesterase, such as eserine, should antagonize the action of HC–3 as it does that of tubocurarine. Evidence for such an effect is shown in the histograms at right on Fig. 2, representative of trials with the first and third fibers of *experiment 9*. Mean miniature epp amplitude increased within 2.5 min after adding eserine to the solution already containing HC–3 for 13 min. Frequencies of miniature epp's appeared to be higher as a result of increased amplitude, since fewer miniature epp's were obscured by base line noise. This experiment greatly reduces the possibility that HC–3 has any presynaptic action under the present conditions: eserine can only amplify miniature epp's, but at this concentration it cannot restore them in the absence of liberated ACh.

c) Lack of effect on quantum content. Results related in the preceding sections demonstrate the occurrence of postsynaptic actions of HC–3 and the unlikelihood of presynaptic ones, such as reduction of quantal size. However, a very sensitive test may be applied to establish if any presynaptic effects take place: estimation from electrical measurements of the number of quanta of ACh released per impulse at an end-plate (13). This method, as opposed to bio-assay, circumvents the possibility of decreased ACh release during rapid stimulation due to an unknown degree of block in nerve branches (Brooks and Thies, unpublished data). The amplitudes of end-plate potentials (epp's) are a function of the amount of ACh reaching postsynaptic receptors as well as of the postsynaptic sensitivity to ACh, both known to decrease during rapid repetitive stimulation (14, 56). Yet, the number of quanta per epp is independent of postsynaptic ACh sensitivity, as miniature epp's and impulse-induced epp's are equally affected (43). Consequently, amounts of ACh released at five single neuromuscular junctions were deduced from intracellularly recorded epp's (Tables 3 and 4).

HC–3 had no detectable effect on ACh release at guinea pig's neuromuscular junctions, as estimated by the quantum content of epp amplitudes. The effects of HC–3 on neuromuscular transmission were compared to those of tubocurarine, a drug known to act postsynaptically. Since choline antagonizes the presynaptic action of HC–3 in sympathetic ganglia (41), observations with HC–3 were also made in the presence of added choline. The results are set forth in Table 3. The three groups of columns in the Table display values of quantum content obtained in three successive tests: at initial slow rates of nerve stimulation (first group), at rapid rates of stimulation (middle group), and, finally, once more at slow rates of stimulation (last group). The 'rapid' rates of stimulation are in the range of natural, continuous, firing rates of motor neurons and of "motor units" of muscle fibers in vivo (1, 24, 27), but they are lower than those occurring during brief reflex activity (1, 21, 22, 24). No evidence is available to determine if ACh release per impulse is maximal under the conditions of such experiments. However, experiments with muscles in vitro reveal that efficacy of neuromuscular transmission suffers above 12/min (10) and, in fact, that ACh per impulse (quantum content) becomes submaximal during stimulation at rates faster than 12/min (57; and Brooks and Thies, unpublished data). Hence, the present experiments were designed to compare "slow" and "rapid" rates of stimulation. Each such sequence of tests, listed by groups of columns and constituting one trial in Table 3, was carried out in the presence of either tubocurarine, HC–3 plus choline, or HC–3 alone. The particular concentrations of drugs used (Tables 3 and 4) were sufficient to prevent contraction of indirectly stimulated serratus preparations without depressing epp amplitudes below accurately measured levels. End-plate potential amplitudes during initial slow stimulation varied between 1 and 13 mv in the experiments shown in Table 3. The trials, listed by number at the beginning of Table 3, appear in the appropriate rows; results of successive trials within experiments,

TABLE 3. *Quantum Content (Reflecting ACh Release per Impulse) at Different Rates of Stimulation*

Expt. No.	Trial No.	Initial Slow Stimulation				Maximum Depression During Rapid Stimulation				Maximum Recovery During Slow Stimulation			
		Stim. freq. (per *min.*)	Drugs used			Stim. freq. (per *sec*)	Drugs used			Stim. freq. (per *min*)	Drugs used		
			d-tubocurarine	HC-3 + choline	HC-3		*d*-tubocurarine	HC-3 + choline	HC-3		*d*-tubocurarine	HC-3 + choline	HC-3
12	1	12	(27)33			5	17			12	30		
	2				(47)39				16				30
	1				110				11				147
13	2	60		117		10		5		6		86	
	3				58				8				62
14	1	72	78			12	19			14	96		
	2	14			77				9				—
15	1	12		187		20		21		12		174	
	2				136				5				57
16	1		27				11				23		
	2	12			25	20			10	12			27
	3		14		(16)		4				8		
	4			14				—				—	

Five neuromuscular junctions were studied, each from a different guinea pig excised serratus preparation; each preparation was stimulated at a slow frequency before and after a period of rapid stimulation. Irrigating solutions contained neuromuscular blocking drugs and increased concentrations of Mg and Ca ions, except for *expt. 14* (values and temperatures shown in Table 4). Dashes show where fibers were lost before completion of all tests. Estimates of quantum content shown in parentheses for the first and last experiments were calculated from epp's recorded about 1 hr before and under the same conditions as those used for initial values. Mean estimates of quantum content had SE of 10–15%.

also identified in the Table, are given in successive rows, reading downwards.

Acetylcholine release per impulse, as reflected in quantum content, was unaffected by the presence of HC–3 during stimulation at slow frequencies (near 12/min, first and last groups of columns in Table 3). Acetylcholine release per impulse generally declined between successive tests, 1–3 hr apart, whether muscle preparations were irrigated with Krebs' solution containing either HC–3, HC–3 plus choline, or tubocurarine. Variations in absolute values of quantum content between the five fibers in Table 3 are due at least in part to alterations in temperature and in concentrations of calcium and magnesium ions (Table 4). These variations between experiments exceed those within experiments where the effects of drugs are compared. Furthermore, successive tests with a single drug also yielded results as widely scattered as those obtained with different drugs. Usually, depression for any one fiber deepened, and recovery became less complete during successive trials (shown one below the other in Table 3). Quantum content increased or decreased by about one quarter after irrigation was changed from Krebs' solution, containing HC–3, to that containing control drugs, or vice versa. Consequently, no action of HC–3 on ACh release per impulse in excess of one quarter of control values has been demonstrated during slow rates of stimulation that yield maximal ACh release per impulse. It will be shown in DISCUSSION that HC–3 would have to depress ACh release by more than $\frac{4}{5}$ in order to block neuromuscular transmission. The diminution of amplitude of electrically recorded potentials can be roughly accounted for by depression of ACh sensitivity. Therefore, any residual presynaptic action, however improbable, such as depression of quantal size of miniature epp's, must be less than the 25 % variation of our data on quantum content.

Does HC–3 have a presynaptic effect on junctions that are firing rapidly, when ACh release per impulse is submaximal and ACh synthesis may be marginal? Results obtained with sympathetic ganglia (41) suggest that this may be so. Evidence against this possibility is presented in the middle group of columns of Table 3. In successive trials ACh release per impulse shows a general deterioration, but no consistent difference is detectable between the extent of maximum depression

TABLE 4. *ACh Release per sec During Rapid Repetitive Stimulation, Calculated from Data in Table 3*

Expt. No.	Trial No.	Temp. (°C.)	Concentrations of Drugs: *d*-tubo-curarine (w/v)	Concentrations of Drugs: HC-3 (M)	Frequency of Stimulation (*per sec*)	Duration of Stimulation (*min*)	No. of Quanta Released per sec in Presence of: *d*-tubo-curarine	No. of Quanta Released per sec in Presence of: HC-3 + choline (2 mM)	No. of Quanta Released per sec in Presence of: HC-3
12	1	24	1.3×10^{-7}	1.1×10^{-5}	5	10	86		
	2					11			78
13	1	28		7.9×10^{-5}	10	8			106
	2					8.5		84	
	3					4			54
14	1	23	3×10^{-7}	2.6×10^{-5}	12	2	229		
	2					2			108
15	1	33		8.0×10^{-5}	20	2		416	
	2					2			98
16	1	33	3×10^{-7}	7.1×10^{-5}	20	4	220		
	2					4			206
	3					4	80		

Irrigating solutions contained neuromuscular blocking drugs and increased concentrations of Mg and Ca ions, except for *expt. 14*. The number of quanta released per sec is the product of frequency of stimulation and quantum content, which usually had a SE of 10% of the mean estimate.

(second group of columns) and of maximum recovery (third group of columns) whether the experiment was carried out with HC–3 or with control drugs, such as tubocurarine or HC–3 plus choline. Thus, during brief rapid stimulation no specific effect of HC–3 on ACh release per impulse was detected.

Further evidence against a specific action of HC–3 on ACh release at neuromuscular junctions is provided by estimates of ACh release per second during rapid repetitive stimulation. Although ACh release per impulse is depressed during rapid rates of stimulation, ACh release per second during such stimulation initially increases to 50–100 times the values obtained during slow rates of stimulation. When rapid stimulation is continued, ACh release per second declines but remains about 10-fold greater than release per second during slow stimulation (Brooks and Thies, unpublished data). ACh release per second is calculated by multiplying quantum content (ACh release/impulse) by frequency of stimulation (impulses/sec), as set out in the last group of three columns in Table 4 (from data in Table 3, column 'Maximum Depression'). Frequencies of stimulation for the same five muscle fibers listed in Table 3 are shown in Table 4, whereas durations of stimulation are listed in the adjacent column, for tests in the presence of control drugs or of HC–3 alone. Although ACh release per second shows some deterioration in successive trials of individual fibers, release is not specifically impaired by the presence of HC–3 (last group of columns in the table). Therefore, continuing release of ACh, as estimated per second during brief rapid stimulation, suggests that ACh synthesis in motor nerve endings persists during short-term experiments in which neuromuscular block is produced by HC–3.

DISCUSSION

1. Effect of HC–3 on Different Tissues

The present results show that HC–3 blocks neuromuscular transmission in guinea pigs by depressing ACh sensitivity, i.e., it acts postsynaptically, much like tubocurarine. Thus, its action at the nerve-muscle junction differs from its effect on cat's sympathetic ganglia where HC–3 depresses ACh synthesis, i.e., it acts presynaptically, presumably by inhibiting choline transport into nerve endings (40). HC–3 depresses miniature epp amplitudes and ACh sensitivity at neuromuscular junctions of the frog's toe muscle in vitro (44, 45), in agreement with the present results, but ACh excitation of the frog's slow rectus muscle is not blocked by HC–3 (49). It has been suggested that HC–3 inhibits ACh synthesis in motor nerve terminals, since posttetanic depression of neuromuscular transmission occurs in HC–3 treated cats, but not in curarized preparations (20). However, interpretation of the in vivo data obtained from one muscle (Fig. 1 of ref. 20) is made difficult by discrepancies in details of procedure between treatments with HC–3 or with curare. For example, continuous slow stimulation was applied to the muscle of the animal given a single dose of HC–3, while the muscle of the curarized animal was allowed to rest. Also, the muscle treated with HC–3 was stimulated more than three times as long as the curarized muscle. Although the results may be due to depletion of ACh content from motor nerve terminals, desensitization to ACh by HC–3 after rapid repetitive stimulation is equally possible: this explanation has been used to explain similar phenomenon in myasthenic muscle (19). Further comparisons of neuromuscular and ganglionic junctions are made in section 3 below.

2. Effects of HC–3 at Various Rates of Stimulation

MacIntosh et al. (42) noted that block of ganglionic transmission by HC–3 was relieved by reducing frequency of stimulation, which is consistent with their demonstration of inhibition of ACh synthesis by HC–3. It has been suggested that deepened depression of neuromuscular transmission upon increasing frequency of stimulation (from 1/10 sec to 1/sec) constitutes evidence for a presynaptic action of HC–3 (49). Yet, the same effect is observed in the presence of tubocurarine (59). It is known that for many postsynaptic blocking drugs (tubocurarine, gallamine, decamethonium, neo-

stigmine, and edrophonium), only one quarter of the dose required for paralysis at slow rates of stimulation, such as 1/sec, is necessary at faster rates of stimulation, such as 5/sec (42). Consequently, greater depression of neuromuscular transmission during more rapid stimulation does not distinguish between presynaptic and postsynaptic actions of HC–3.

3. Interaction of HC–3 with Other Drugs

Several attempts have been made to determine the site of action of HC–3 by testing the antagonism of other drugs. The in vivo toxicity of HC–3 to mice is counteracted by eserine or choline (51), and neuromuscular block in rabbits is counteracted by neostigmine or choline (49). In the present experiments with guinea pigs eserine was ineffective in preventing death, but it did partially reverse neuromuscular block. Species differences may account for the discrepancy in toxicity. These results are compatible with each other and with a postsynaptic site of HC–3 action, since eserine and neostigmine are well-known antagonists of tubocurarine and of similar drugs (17). Eserine does not depress ACh synthesis in nerve-muscle preparations (53) as it does in sympathetic ganglia; this depression was attributable there to loss of choline (47). Finally, the reversal of HC–3 block of neuromuscular transmission in rabbits by choline (49, 50) need not implicate a presynaptic action of HC–3 (40, 41) as tubocurarine block in cats can be relieved by the same dose of choline (28).

Differences between rates of utilization of choline in cat sympathetic ganglia and guinea pig muscles, as well as differences in choline content, may account for the lack of action of HC–3 on ACh synthesis in the present experiments. The supply of choline appears to limit ACh synthesis in superior cervical ganglia, whereas it does not do so at neuromuscular junctions. ACh output from perfused sympathetic ganglia is increased by addition of choline to the perfusion fluid (9), and choline is rapidly utilized by the cat's superior cervical ganglion: more than a quarter of the choline added to perfusing blood plasma is removed in one trip through the ganglion (40). In contrast, the addition of choline to Krebs' solution irrigating rat diaphragm fails to increase ACh output (53). If sufficient choline is present in guinea pig motor nerve terminals to maintain maximal ACh synthesis, then HC–3 would not likely depress such synthesis by inhibiting choline transport. This may indeed be the case: guinea pig's muscle contains five times as much choline as rat's or rabbit's muscle (30) and guinea pig blood contains ten times as much choline as cat's or rat's blood, and five times as much as rabbit's blood (2, 29).

4. Toxicity of HC–3 and Doses Required for Neuromuscular Block

The estimated LD_{50} of HC–3 for guinea pigs (0.5 mg/kg), similar to that for rats (0.45 mg/kg; ref. 52), fits the relationship suggested by Schueler (51): decrease of HC–3 toxicity as size of species increases. Choline antagonizes HC–3 toxicity less effectively for guinea pigs than for mice, whereas eserine is ineffective (Table 1). Although choline completely counteracts ten times the LD_{50} of HC–3 for mice (51), it is completely effective only at doses of HC–3 that are double the LD_{50} for guinea pigs. The high concentrations of free choline present in guinea pig blood and muscle may be responsible for this difference. Thus, on the one hand choline, but not eserine, can counteract HC–3 toxicity for guinea pigs. On the other hand eserine, but not choline, can reverse HC–3 neuromuscular block of in vitro preparations. These results suggest that neuromuscular block is probably not responsible for HC–3 toxicity in guinea pigs.

Schueler (51) suggested that neuromuscular block is not responsible for HC–3 toxicity in a variety of animals, since 10–30 times the toxic dose of HC–3 was required for rapid respiratory failure and what appeared to be neuromuscular paralysis. Data from subsequent studies of the action of HC–3 on nerve-muscle preparations in vivo, as well as in vitro, substantiate his suggestion. Injection of 20 times the LD_{50} of HC–3 into artificially respired rabbits causes extreme depression of contractions in slowly stimulated gastrocnemius muscles (49). In dogs transmission to slowly stimulated muscles fails with about ten times the toxic dose of HC–3 (51, 52), whereas in cats 3–6 times the toxic dose suffices to block transmission in rapidly stimulated muscles (32). Slowly stimulated muscles of cats would be expected to require higher doses of HC–3 for neuromuscular block (cf. ref. 48). Comparisons between doses causing neuromuscular block in vitro and HC–3 toxicity are more indirect. Yet, since Domer (1959, in ref. 52) showed that half an injected dose of HC–3 remains in the blood of rats (8% of body weight), 1 mg/kg of HC–3 in vivo would be equivalent to approximately 6 mg/liter of blood in vitro. Consequently, injection of guinea pigs with the LD_{50} (0.5 mg of HC–3/kg) may result in a concentration of 3 mg/liter of blood. More than ten times this dose, 6×10^{-5} M HC–3 or 35 mg/liter, is required to produce severe block of neuromuscular transmission in vitro (Fig. 1). Therefore, neuromuscular block occurs at ten times the lethal dose of HC–3 in vitro as well as in vivo. The only effect observed in vitro with doses equivalent to the LD_{50} (0.5 mg HC–3/kg or 6×10^{-6} M) was depression of miniature epp amplitude to less than half of control values (second fiber, *Expt. 9*). Similar depression of response to released ACh at all neuromuscular junctions would be expected to cause little impairment of muscular activity since epp's rise to more than three times threshold (3). The minor effect of toxic doses of HC–3 on neuromuscular transmission is additional evidence that neuromuscular block may not be responsible for HC–3 toxicity in guinea pigs. It must be borne in mind, however, that these data regarding quantum content (Tables 3 and 4) were obtained after only a few thousand nerve volleys had gone through the junctions. Muscles poisoned in situ may undergo more vigorous activity.

5. Comparison of the Actions of HC–3 with Other Neuromuscular Blocking Agents

It has been shown in RESULTS that HC–3 blocks neuromuscular transmission by depressing postsynaptic sensitivity to ACh. Considerations have been presented above of the toxicity of HC–3 and its effects on different tissues, at various rates of stimulation, when acting alone or in combination with other drugs. It is now possible to characterize the neuropharmacology of this drug with regard to the nerve-muscle junction.

a) Presynaptic actions

NERVE BLOCK. HC–3 never impaired the conduction of nerve impulses, since postsynaptic responses to every stimulus were recorded at single neuromuscular junctions (expts. shown in Tables 3 and 4). In contrast, anoxia causes failure of one-to-one conduction in a contracting muscle subjected to rapid, indirect stimulation (34, 35; and Brooks and Thies; unpublished data); such failure also occurs during slow stimulation in the absence of glucose (Thies, unpublished data). Furthermore, indirect excitability was maintained in the present experiments during stimulation of the nerve in the Krebs' bath containing HC–3 (*expt. 3*). Apparently HC–3 has no action similar to procaine, which blocks nerve activity by increasing the threshold required for excitation (58).

ACh SYNTHESIS. The present studies show that HC–3 most likely has no action on ACh synthesis at neuromuscular junctions, since ACh release in vitro continues for many minutes in the presence of concentrations of HC–3, that may be ten times those causing death in vivo (Table 4). However, inhibition of ACh synthesis cannot be excluded completely by our results, since maintenance of the ACh content was only deduced from continuing ACh release (Table 4). Previous experiments that suggested such an action (49) are inconclusive: dependence of block upon frequency of stimulation and antagonism of block by choline are also seen in the presence of tubocurarine (28, 59). However, inhibition of ACh synthesis in sympathetic ganglia by HC–3 (41, 42) produces failure of transmission and of ACh output, similar to that seen in the absence of glucose (31, 37). Although glucose is required for neuromuscular transmission (25), lack of glucose blocks nerve conduction before any effect upon ACh synthesis is detectable (Thies, unpublished data). HC–3 lacks general effects on ACh metabolism; for instance, it does not inhibit ACh synthesis in *Lactobacillus plantarum* or in isolated guinea pig's ileum (Long, personal communication).

ACh RELEASE. In our experiments, release of wash during slow stimulation in the presence of HC–3 AC similar to ACh release in the presence of tubocurarine or of HC–3 plus choline. In agreement with this result, no effect of HC–3 was observed on ACh release at frog neuromuscular junctions (44, 45). Table 3 shows that quantum content, reflecting ACh release, varied by one quarter when either drug was used. Release would have to be depressed by more than four fifths before mammalian neuromuscular junctions would be blocked, since it may be shown that normal epp's, which rise to three times threshold (3), represent release of more than five times the ACh required for threshold responses (43). ACh release is severely depressed, if not abolished, by botulinum toxin (5, 6), by procaine (53a), by high concentrations of magnesium ion (12), or by low concentrations of calcium ion (16). ACh release per impulse is decreased about 30-fold by increasing the concentration of magnesium ions ten times in frog (13), cat (3), or rat (38). In contrast, quantum content at frog neuromuscular junctions is unaffected by tubocurarine (43).

The paucity of evidence for presynaptic actions of HC–3 at guinea-pig motor nerve terminals distinguishes HC–3 block from that caused by anoxia, lack of glucose, botulinum toxin, procaine, low concentrations of calcium ion, or high concentrations of magnesium ion.

b) Postsynaptic actions

EXCITABILITY OF MUSCLE MEMBRANE. There is no evidence that HC–3 raises the threshold for excitation of postsynaptic membranes, as do general anesthetics or high concentrations of calcium and magnesium ions (12, 36, 46, 55). The high concentrations of magnesium and of calcium ions routinely added in *experiments 12, 13, 15* and *16* (Tables 3 and 4) permitted recording of epp's as large as 9–13 mv without propagated activity, whether or not HC–3 was present. The threshold for excitation was not tested by direct stimulation from within single fibers, but the diaphragm muscle was directly excitable in the presence of HC–3 (Fig. 1).

HC–3 does not appear to depolarize the membrane, even transiently. Although no systematic tests were performed, resting membrane potentials remained constant in *experiments 2, 3* and *9* during depression or obliteration of miniature epp amplitudes. Thus there is no analogy between the actions of HC–3 and those of decamethonium (11, 54).

ANTICHOLINESTERASE ACTIVITY. HC–3 has only very slight action on commerically prepared bovine erythrocyte cholinesterase (39).

ACh SENSITIVITY. The present experiments show that the neuromuscular block of guinea pig muscles is caused by depression of ACh sensitivity. Similarly, epp amplitudes are decreased at single frog junctions treated with HC–3, without concommitant decreases of ACh release or of membrane resistance (44). This type of block is characteristic of tubocurarine, although it also occurs with decamethonium (54), procaine (23, 26), pentobarbital (55), and high concentrations of magnesium ions (12). Part of our evidence rests on the demonstration that HC–3 depresses sensitivity to applied as well as to released ACh, since higher concentrations of ACh are required to elicit twitches in the presence of HC–3 (Table 2). Such results must be treated cautiously because, in the presence of agents that depress electrical excitability, greater concentrations of ACh would be required to cause the larger responses necessary to initiate propa-

gated activity; as for instance in the case of depression of ACh sensitivity by pentobarbital (55). However, in the present case there is no evidence for elevation of thresholds to electrical stimulation. Furthermore, non-propagating responses to ACh applied at single junctions are decreased by HC–3, again demonstrating depression of ACh sensitivity per se (44, 45). Finally, the antagonism of the action of HC–3 by anticholinesterase drugs (Fig. 2, Table 2; ref. 49), which permits ACh to accumulate at junctions, further demonstrates the similarity between the actions of HC–3 and of tubocurarine at the neuromuscular junction.

SUMMARY

HC–3 blocks neuromuscular transmission in resting or active guinea pigs' excised nerve-muscle preparations.

The sensitivity of end-plate regions of muscles to externally applied acetylcholine is decreased to less than 1 % of normal by blocking doses of HC–3.

HC–3 depresses the amplitude of spontaneous miniature end-plate potentials without independently changing their frequency.

The effects of HC–3 on neuromuscular transmission, miniature end-plate potentials, and ACh sensitivity are partially reversed by eserine, but not by choline.

Variations of ACh release, as estimated by quantum content, are similar in the presence of HC–3 to those observed in the presence of tubocurarine or of HC–3 plus choline.

These experiments demonstrate postsynaptic actions of HC–3 at this mammalian neuromuscular junction in vitro. No presynaptic actions were found.

The toxicity of HC–3 to guinea pigs is antagonized by choline, but not by eserine. Toxicity may not be due to neuromuscular block in this species.

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Postsynaptic action of HC-3 on neuromuscular transmission[1]

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HEMICHOLINIUM NO. 3 (HC–3) has been shown to interfere with cholinergic transmission at several sites in the peripheral nervous system. In particular, the experiments of MacIntosh *et al.* (7, 8) provided evidence that the principal action of the compound in perfused sympathetic ganglia is to reduce acetylcholine (ACh) synthesis. Subsequently, Reitzel and Long (13) demonstrated that HC–3, in doses of the order of 0.2 mg/kg, produced neuromuscular block during repetitive nerve stimulation in vivo, provided the frequencies of stimulation were greater than 1 per sec. The effects of the compound were antagonized by choline in both cases, and it was concluded that its action at the neuromuscular junction was similar to that at the ganglion. However, similar dependence of neuromuscular block on stimulus frequency has been observed by Preston and Van Maanen (12) with a variety of postsynaptic blocking agents, and Hutter (6) has shown that neuromuscular block produced by curare may be relieved by choline.

The experiments to be presented here and those of Brooks and Thies (1, 2) indicate that the compound has, in common with other quaternary ammonium ions, a postsynaptic blocking action at the neuromuscular junction of both frogs and mammals. It was observed by Brooks and Thies that this action was not antagonized by choline, and the present experiments indicate that when neuromuscular transmission is blocked by HC–3, the end-plate potential (epp) is, in fact, further reduced in amplitude by the addition of choline to the bathing solution. These results are probably analagous to the effects observed by Reitzel and Long with higher doses of HC–3 (about 5 mg/kg). Some of the present results have been presented previously (10, 11).

METHODS

The usual techniques were employed for intracellular recording of epp's, for the production and recording of electrotonic potentials, and for the iontophoretic application of ACh to the end-plate regions of muscle fibers (4, 5). The m.ext.l. dig. IV of the frog (*Rana pipiens*) was dissected with its nerve supply intact and immersed in Ringer's solution of the following composition: 115 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$. The end-plate response was reduced below threshold for the initiation of a propagated action potential either by adding 0.5–3.0 $\times$ 10^{-6} M *d*-tubocurarine (DTC) to the bath, or by replacing an osmotically equivalent amount of NaCl with 8–12 mM $MgCl_2$. Nerve stimulation was restricted to less than six shocks per minute.

Most of the experiments with choline were performed by recording extracellularly from the end-plate region of the isolated frog sartorius muscle with a platinum electrode, an indifferent electrode being in contact with the pelvic end of the muscle. Results so obtained were confirmed by intracellular recording, as described above, or by similar intracellular recording from the tenuissimus muscle of the cat.

RESULTS

Postsynaptic blocking action of HC–3. The records in Fig. 1 *A* were obtained from a neuromuscular junction blocked by the addition of 8 mM Mg to the bathing solution. Those in *B* were obtained from the same junction after the addition of 3 $\times$ 10^{-4} M MC–3, the Mg concentration remaining unchanged. Upon the addition of HC–3 the amplitude of the epp was reduced, and its falling phase greatly prolonged. In both *A* and *B* the first record is the largest, the third is the smallest, and the second is the mean of the series from which they were obtained. Although the number of epp's in each series was small (30–50), an estimate of the coefficient of variation of the amplitude distribution was obtained. This is a measure of the variation in amplitude of successive epp's, and is given simply by the standard deviation divided by the mean. An increase in coefficient of variation would indicate a decrease in mean quantum content of the epp's (3, 9). As suggested by the figure, the relative spread of the amplitudes, and hence the coefficient of variation, appeared unchanged, indicating that the quantum content was unaltered by the addition of the HC–3. This was confirmed in one experiment in which 200 epp's were recorded from a single junction, both before and after the addition of HC–3 to the bath. Although the mean amplitude of the epp's was approximately

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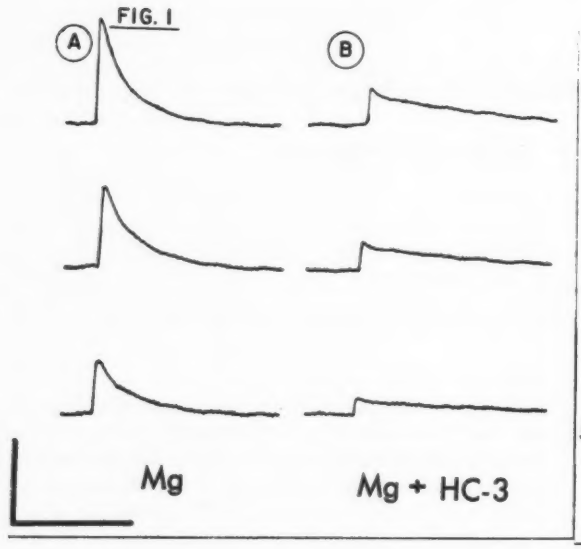

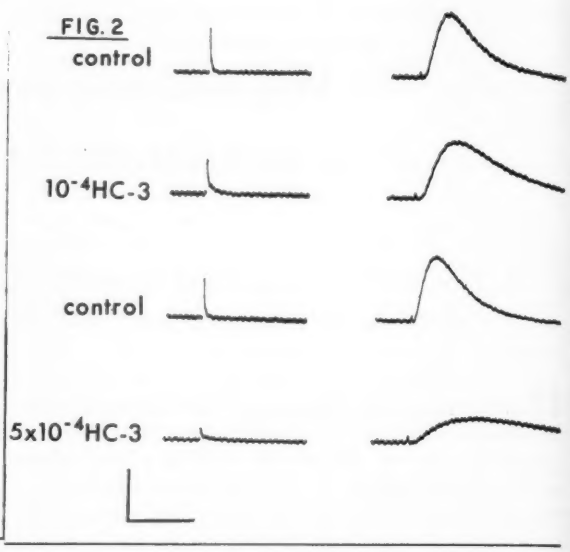

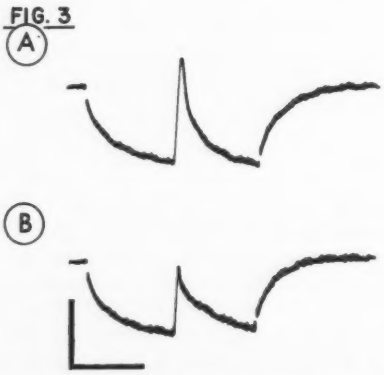

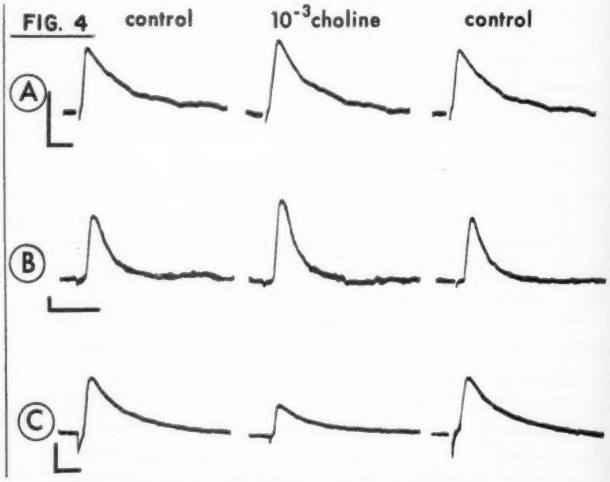

FIG. 1. Effect of HC-3 on amplitude and time course of epp's recorded from a fiber in which neuromuscular transmission was blocked by increasing Mg concentration in bathing solution to 8 mM. *A:* control records; *B:* 3×10^{-4}M HC-3 added to bath. Note reduction in amplitude and prolongation of falling phase. Calibrations: 5 mv, 50 msec.

FIG. 2. End-plate potentials (first column) and ACh potentials (second column) recorded from a single junction blocked with 10.5 mM Mg. Note reduction in amplitude of both potentials and prolongation of ACh potential as HC-3 concentration is increased. Calibrations: 5 mv, 0.2 sec.

FIG. 3. Effect of HC-3 on epp and electrotonic potential. *A:* neuromuscular transmission blocked with 7×10^{-7}M DTC, electrotonic potential produced by passing $4\text{-}5 \times 10^{-8}$A through the membrane. Electrode separation 45 μ. *B:* 10^{-4}M HC-3 added to bath, all other conditions unchanged. Epp amplitude is reduced, electrotonic potential unaltered. Calibrations: 5 mv, 50 msec.

FIG. 4. Effects of choline on neuromuscular transmission. *A:* extracellular records from end-plate region of frog sartorius muscle; transmission blocked with 2.8×10^{-6}M DTC. Control records taken before addition and after removal of 10^{-3}M choline to the bathing solution. Note slight increase in amplitude when choline is present. *B:* similar series recorded intracellularly from a single fiber of the tenuissimus muscle of the cat. Amplitude increase is more marked than in *A*. *C:* extracellular records from frog sartorius; transmission blocked with 10.5 mM Mg. Note reduction in epp amplitude in presence of choline. Calibrations: 1 mv, 10 msec.

halved in the presence of HC–3, the quantum content remained unchanged.

The lack of any change in quantum content suggests that the action of the drug is on the end-plate receptors rather than on the nerve terminals. However, these results are far from conclusive, since the possibility of a reduction in the size of the quantum, or "packet" of ACh has not been ruled out. Indeed, the production of such a change in "packet" size by a compound known to interfere with ACh synthesis would hardly be surprising. A more crucial test of its site of action is to determine whether it produces any change in sensitivity of the

motor end-plate to applied ACh. Such an experiment is illustrated in Fig. 2. Records were taken simultaneously of the epp and the "ACh potential" produced by the iontophoretic application of ACh to the end-plate from a micropipette. Neuromuscular transmission was blocked by increasing the Mg concentration in the bath to 10.5 mM. In the first control the epp amplitude was about 4 mv, and the amplitude and duration of the current pulse through the ACh electrode was adjusted to give an ACh potential of similar magnitude (currents used were of the order of 5×10^{-7} A for about 5 msec). When 10^{-4} M HC–3 was added to the bath there was a slight reduction in the amplitudes of both potentials. In addition, the durations of both the ACh potential and the falling phase of the epp were prolonged. On washing out the HC–3, both potentials returned to their original size and time-course. Increasing the HC–3 concentration to 5×10^{-4} M produced a more marked reduction in amplitude and a greater effect on the time course of both potentials. The results of three such experiments are summarized in Table 1. In each case the reduction in amplitude of the epp is similar in magnitude to that of the ACh potential.

Before concluding that the observed action of HC–3 is on the motor end-plate it is necessary to show that it does not have a more general effect on the muscle fiber membrane. For example, the observed reduction in epp amplitude might be due to a decrease in membrane resistance. This possibility was tested by inserting a second micropipette into the fiber and passing rectangular current pulses through the membrane. The resulting electrotonic potentials were recorded before and after the addition of HC–3. For purposes of illustration, epp's were recorded simultaneously as shown in Fig. 3. Although the concentration of HC–3 used was sufficient to reduce the amplitude of the epp by about 40%, there was no effect on the electrotonic potential, indicating that the passive electrical properties of the fiber were unaltered.

Effects of choline. The absence of any reversal by choline of neuromuscular block produced by HC–3 observed by Brooks and Thies (2) is in contrast with the results of Reitzel and Long, and those obtained by Hutter in preparations blocked by curare. In an attempt to determine the nature of the discrepancy, the effects of choline on epp amplitude were examined in muscles blocked with DTC, Mg, and HC–3. Extracellular records from the motor end-plate region of the sartorius muscle of the

TABLE 1. *Effect of HC-3 on Amplitudes of epp's and ACh Potentials*

Fiber	Control		HC-3			% Reduction	
	$\bar{e}$* (mv)	$\bar{V}$† (mv)	Concn. (M)	$\bar{e}$ (mv)	$\bar{V}$ (mv)	$\bar{e}$	$\bar{V}$
1	6.1	4.1	1×10^{-4}	5.4	3.5	11	15
1	6.1	4.1	5×10^{-4}	2.4	1.8	60	56
2	6.6	2.5	1×10^{-4}	3.8	1.4	43	44
3	5.1	2.5	3×10^{-4}	1.2	0.6	76	76

* Mean amplitude of epp. † Mean amplitude of ACh potential.

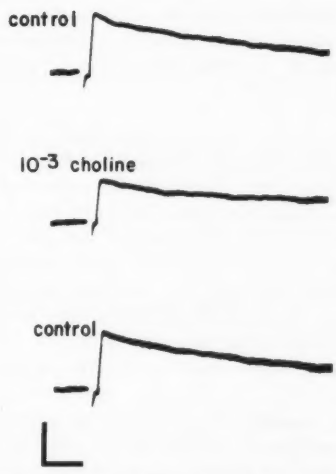

FIG. 5. Effect of choline in presence of HC-3. Extracellular records from frog sartorius. Transmission blocked by addition of 5×10^{-4}M HC-3 to bathing solution, plus 4 mM Mg. Epp amplitude is reduced by addition of 10^{-3}M choline. Calibrations: 1 mv, 10 msec.

frog are shown in Fig. 4 *A*. Neuromuscular transmission was blocked by the addition of 2.8×10^{-6}M DTC to the bathing solution. Bathing the preparation for 20 min in the same solution containing, in addition, 10^{-3} M choline produced a small (about 10%) but consistent increase in epp amplitude. On washing out the choline, the amplitude returned to the previous control value. These results are typical of those obtained with extracellular recording from a large group of end-plates. When recording intracellularly from the end-plate regions of single fibers, amplitude changes ranged from zero to increases of greater than 30%. One such large increase is shown in the records of Fig. 4 *B*, taken from a single fiber in the tenuissimus muscle of the cat. These results are in sharp contrast with those obtained when neuromuscular transmission is blocked by 10.5 mM Mg. As the extracellular records of Fig. 4 *C* indicate, the addition of choline always produced a decrease in epp amplitude. The experiments reported here are in agreement with those of Hutter (6), and illustrate the dual action of choline at the neuromuscular junction: *a*) it increases the amount of transmitter reaching the end-plate receptors, and *b*) it acts as a postsynaptic blocking agent. The second action is apparently dominant, except when the end-plate receptors are "protected" by the presence of another postsynaptic blocking agent such as DTC.

Fig. 5 illustrates the effect of choline on epp amplitude when neuromuscular transmission is blocked by 5×10^{-4} M HC–3. Since complete block was not obtained with the compound, a small amount of Mg (4 mM) was added to the bathing solution. Again, the addition of choline resulted in a decrease in epp amplitude, the effect being indistinguishable from that observed in the Mg-blocked preparation. It appears, then, that although the block of neuromuscular transmission produced by

HC–3 in these concentrations is postsynaptic, the compound is ineffective in protecting the neuromuscular junction from the additional blocking action of choline.

DISCUSSION

These results, together with those of Brooks and Thies, clearly demonstrate a postsynaptic action of HC–3 at the neuromuscular junction. They are not, however, in conflict with previous reports that the principal action of the drug is on ACh synthesis. Such an action would not have been revealed by the present experiments, since the low frequencies of stimulation would not be expected to deplete the ACh stores during the period of an experiment, particularly in the Mg-treated preparations.

Since the neuromuscular blocking action observed by Reitzel and Long with low doses of HC–3 was antagonized by choline, their conclusion that the effect was not postsynaptic would appear to be correct. As shown by the present experiments and by those of Brooks and Thies, choline does not antagonize the postsynaptic block produced by HC–3. On the other hand, failure of neuromuscular transmission due to inhibition of ACh synthesis might conceivably be reversed by choline, in spite of its own postsynaptic blocking action.

SUMMARY

Effects of hemicholinium No. 3 (HC–3) on neuromuscular transmission have been studied, using both intracellular and extracellular recording techniques on isolated frog's nerve-muscle preparations. Concentrations of HC–3 greater than 10^{-5} M decreased the amplitudes of end-plate potentials and potentials produced by iontophoretic application of acetylecholine to the neuromuscular junction. Since the magnitude of the effect is similar for both potentials, it is concluded that in these concentrations HC–3 acts on the motor end-plate rather than on the nerve terminals. This postsynaptic action is not antagonized by choline.

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Hemicholiniums: structure activity relationships and actions on the peripheral nervous system[1]

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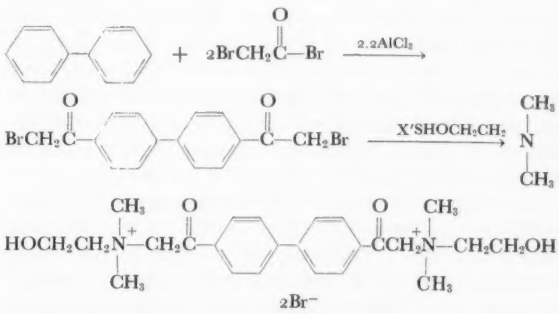
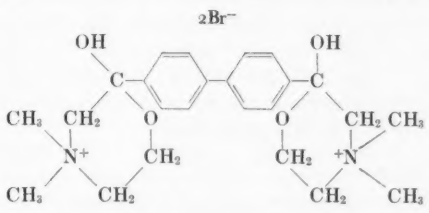
LONG AND SCHUELER (8) reported a series of biphenyl derivatives that were potent cholinesterase inhibitors. One of the compounds, which is referred to subsequently as hemicholinium (HC–3), was noted to be an extremely toxic material, following intraperitoneal injection in mice. Even though this substance was an analogue of compounds showing very prominent cholinesterase inhibition, it was found to lack this activity. The compound was reported originally to be devoid of action at the neuromuscular junction (8) in experiments using the sciatic nerve gastrocnemius muscle preparation in the cat. The nerve was stimulated once every 10 sec using single shock. Studies, to be discussed later in this paper, demonstrate that hemicholinium does have activity at the neuromuscular junction when higher frequencies of stimulation are used. The compound also has been demonstrated by MacIntosh and co-workers (10–12) to reduce the amount of acetylcholine in a perfused ganglia as well as virtually to deplete the acetylcholine in the perfusate obtained from the ganglia. More recent studies indicate that the compound also produces transmission failure at the postganglionic parasympathetic and sympathetic nervous systems. The mechanism of action of hemicholinium-like compounds remains to be further clarified, but the work of MacIntosh and co-workers suggests that these compounds may interfere with the synthesis of acetylcholine, or that they may be involved in preventing the release of acetylcholine from the nerve terminals at cholinergic sites. The inhibitory effect on the sympathetic nervous systems probably involves the nerve terminal. Hemicholinium (HC–3) appears to be an unusual compound, in that it can produce inhibition at all sites of transmission in the peripheral nervous system as well as various actions in the central nervous system (7, 17, 18).

CHEMISTRY OF HEMICHOLINIUM

Long and Schueler (8) reported the synthesis of the original compound and the synthesis is shown below:



The compound was reported originally as the open chain type structure shown above. Schueler (18) demonstrated that the compound undergoes a hemiacetal type rearrangement and the final structure is shown below:



On the basis of this structure Schueler (18) suggested the name hemicholinium. From a chemical point of view this is a rather unusual structure. Although rearrangements with tertiary analogues have been reported, this is perhaps the first report of this rearrangement involving a quaternary.

The material is obtained as a white cystalline solid and, depending on the solvent used for crystallization, the substance shows two different melting points, one at 180°C and the other at 220°C. These different materials may represent different isomers that are possible or possibly different crystalline forms. However, biological assay indicates no difference in the activity of these two compounds.

ANALOGUES OF HEMICHOLINIUM

When the dimethylaminoethanol moiety is replaced by pyridine or picoline, compounds are obtained that

[1] Supported in part by a grant from the U. S. Public Health Service Grant No. B-1396.

possess as much as 100 times the activity of neostigmine methylsulfate when evaluated as inhibitors of bovine erthrocyte cholinesterase (8). These compounds do not demonstrate the myoneural inhibiting properties observed with HC–3. Schueler (18) evaluated various alkyl, alkanol, and heterocyclic analogs, and the most active compounds were the dimethyl or diethylaminoethanol derivatives. He also reported that the phenacyl analogue (½ of the HC–3) was inactive.

Marshall and Long (13) have reported on various analogues of hemicholinium. These structural modifications have involved both alterations in the cationic heads as well as modification of the biphenyl nucleus. These investigators reported a series of compounds in which both the cationic head and biphenyl nucleus were altered. The biphenylmethane, biphenyl ether, and benzidine analogues of hemicholinium were observed to be less active. It was demonstrated that with these agents there was an increase in cholinesterase inhibiting activity in comparison with the parent compound. Biphenyl analogues of pyridyl-2-carbinol possessed both cholinesterase inhibiting and HC–3-like activity.

Powers *et al.* (14) have synthesized recently three new aliphatic analogues of HC-3 which were bisamino derivatives of *n*-decane-2-9-dione. The amino substitutions were with trimethylamine, dimethylaminoethanol, and pyridine. Only the alkanol derivative demonstrated HC-3-like activity. Therefore, it appears that the original compound is still the most active of the series of substances having a hemicholinium action. Further studies are required, if one hopes to obtain compounds that are more selective in their site of action.

Therapeutic application, of course, might be possible for compounds relatively selective for the somatic nervous system. Selective blockade of parasympathetic or sympathetic nervous system or both, would also offer interesting therapeutic possibilites.

TOXICITY

Intravenous toxicity in animals has been studied in a variety of species. In mice, the LD_{50}'s reported vary from 20–75 μg/kg. In larger species of animals the compound appears to be less toxic. The LD_{50} in rabbits in 260 μg/kg. In cats the approximate LD_{50} is 300 μg/kg., and in the dog, 750 μg/kg. The appearance of signs of toxicity is interesting in laboratory animals. In all species the onset of action is rather slow. For example, rabbits appear normal for about 10–15 min following the intravenous administration of the compound. Then there is a gradual depression of respiration and difficulty in locomotion. If the animals have received a lethal dose of the compound, death usually occurs in about 45 min following the administration of the compound. If the animal survives, recovery is rather slow and 2 to 3 hours are required before the animals appear normal. In larger species of animals, for example, the dog, the onset of action is even slower and the time for death is much more delayed than it is for the smaller species. Giovinco (6) demonstration that motor activity influenced the LD_{50} in mice. Increasing motor activity in the animals increased the LD_{50}. Depression of the animals by pretreatment with barbiturates decreased the LD_{50}.

ANTIDOTES FOR TOXICITY

Schueler *et al.* (17) and Schueler (18) reported that choline was an effective antagonist for hemicholinium poisoning. Reitzel and Long (15, 16) confirmed this original report and studied a series of choline analogues for their ability to antagonize hemicholinium toxicity. Various analogues of choline were shown to be ineffective as antagonists to hemicholinium induced toxicity, although various choline esters were antagonistic. However, if the animals were pretreated with a cholinesterase inhibitor, thus preventing the hydrolysis of choline ester, the choline esters did not antagonize hemicholinium toxicity. Therefore, it was suggested that the choline moiety is relatively specific as an antidote. Choline was capable of preventing death in dogs that received up to 60 mg/kg of hemicholinium. Giovinco (6) reported that physostigmine had some antagonistic effect against hemicholinium toxicity. This marked antagonism by choline is perhaps suggestive that these compounds may be acting by interference with the choline portion of the cholineacetylase system, or perhaps interfering with choline transport into the enzyme system.

ACTION AT THE MYONEURAL JUNCTION

As noted in the introduction, the hemicholiniums do not produce failure in transmission when somatic nerves are stimulated at slower frequencies. When the nerves are stimulated with fast frequencies or with interrupted tetanic stimulation, the hemicholinium is an extremely active neuromuscular blocking agent (9, 15). At a frequency of stimulation of 1 per sec, the ED_{50} for neuromuscular blockade in the rabbit sciatic nerve-gastrocnemius muscle preparation is approximately 40 μg/kg. The onset and duration of action closely parallels observable signs in unanesthetized rabbits reported above in the section under toxicity. When the frequency of single shock stimulation is 1 per sec, the latent period was approximately 10–15 min, following the intravenous administration of hemicholinium. Maximal blockade occurs in 40–60 min after the injection, followed by a gradual return back to control contractions. With interrupted tetanic stimulation the activity of the compound was increased markedly. Using interrupted tetanic stimulation with a frequency of 250 per sec, the ED_{50} was approximately 3 μg/kg. Under these conditions the duration of action was much longer and often the preparation required at least 7–8 hr to return to control levels. Additional studies indicated that very large doses, 2–4 mg/kg, produced almost immediate neuromuscular blockade. The blockade produced by these large doses was reversed readily by neostigmine bromide,

hence indicating that the mechanism involved was a "curare" type of action. Choline was observed to be a very effective antagonist at the neuromuscular junction. The higher alkyl analogues of choline were observed to be relatively poor, or inactive, as antagonists to hemicholinium-induced neuromuscular blockade (16).

Hemicholinium was evaluated using the sciatic nerve-gastrocnemius muscle preparation in the chicken. In this preparation the compound decreases induced contractions, and the response looks quite similar to that obtained with *d*-tubo-curarine chloride. However, the contracture induced by the intravenous administration of acetylcholine is not antagonized by hemicholinium. This is further evidence that the mechanism of action is different from that of the classical competitive neuromuscular blocking agents. Hemicholinium in concentrations up to 10^{-3}M did not produce contraction of the frog rectus abdominus muscle, neither was there antagonism or potentiation of the contracture obtained with acetylcholine (15). This is additional evidence that the mechanism of action is different than the classical neuromuscular blocking agents. Desmedt (2, 3) has demonstrated that HC-3 produced "posttetanic exhaustion" in cats anesthetized with chloralose. This effect closely resembles the "posttetanic exhaustion" observed in myasthenic patients, an effect different from that observed with curare. Gesler *et al.* (5) have reported a series of compounds that possess properties similar to those discussed above.

ACTION ON THE PARASYMPATHETIC NERVOUS SYSTEM

Wilson and Long (19) reported that the compound was effective in blocking saliva formation following stimulation of the chorda tympani nerve. The ED_{50} in this preparation was approximately 10 μg/kg. With higher frequencies of stimulation, the compound became more effective in producing transmission failure. Intravenous administration of postganglionic parasympathetic stimulants, such as acetyl β-methylcholine, produced as much saliva, following hemicholinium, as was produced prior to the administration of the compound. In this respect, the action on the parasympathetic nervous system appears to be similar to that discussed above for the somatic nervous system. The muscarinic receptors appear to be unaltered by the compound. On these preparations the antagonistic action of choline was not as definite as discussed above for the myoneural junction. The large doses of choline required to antagonize HC-3 were of such magnitude that direct muscarinic effect could have been the explanation of the increased formation of saliva. Unpublished observations from our laboratory show that the compound possesses similar activity in blocking contractions following vagal stimulation of the small intestine and colon, in blocking the contraction of the urinary bladder, following pelvic nerve stimulation, and in blocking the miotic effects of preganglionic or postganglionic ciliary nerve stimulation. It was also observed that topical application of HC-3 to the eye produced mydriasis. The pupillary response is similar to that observed with atropine sulfate, but in mice HC-3 is about $\frac{1}{100}$ as active as atropine. However, the postganglionic parasympathetic stimulating agents are effective in producing miosis, following maximal mydriasis induced by hemicholinium. In a series of ten rabbits it was observed that hemicholinium was capable of lowering intraocular pressure in these animals, in contrast to the increase in intraocular pressure that one observes following the instillation of atropine sulfate. Chang and Rand (1) demonstrated that HC-3 was capable of blocking the response to nerve stimulation in various isolated organ preparations. HC-3 produced inhibition in the vagus-isolated rabbit atria and the parasympathetic innervation of the isolated rabbit vagina.

GANGLIA

MacIntosh and co-workers (10–12) have demonstrated that HC-3 added to the perfusing solution depletes the acetylcholine found in a perfused cervical sympathetic ganglia. Wilson and Long (19), using in vivo experiments, failed to observe inhibition of the splanchnic nerve-adrenal gland preparation. With the nictitating membrane preparation of the cat, HC-3 rendered the membrane unable to remain in a contracted state during nerve stimulation.

POSTGANGLIONIC SYMPATHETIC NEUROEFFECTOR JUNCTION

Chang and Rand (1) demonstrated inhibition of the electrical stimulated sympathetic nerves in the following preparations: vas deferens, rabbit uterus, rabbit colon, perfused rabbit ear, and isolated cat atria. Choline was partially effective in antagonizing the inhibition in these preparations. Chang and Rand (1) have suggested that their results may support their hypothesis that acetylcholine may be involved in the liberation of norepinephrine. In this laboratory we have observed inhibition in vivo of contractions of the urinary bladder induced by hypogastric nerve stimulation. Also, there is inhibition of postganglionic sympathetic nerve stimulation in the dog fore limb not accompanied by an inhibition of vasoconstriction produced by tyramine. In some respects, the effect of HC-3 on the sympathetic nervous system resembles bretylium, but the mechanism of action remains to be established.

MISCELLANEOUS OBSERVATIONS

MacIntosh (10) and Gardiner (4) have reported that HC-3 produces inhibition of rat brain cholineacetylase. Lactobacillus plantarum have been demonstrated to have the capacity to synthesize acetylcholine. Various studies on this bacteria, in this laboratory, have failed to demonstrate any inhibition of acetylcholine synthesis. The compound in concentrations of approximately 10^{-5} M has the ability to produce degeneration in planaria. It has approximately the same activity as atropine sulphate in producing degeneration. In concen-

trations of approximately 10^{-5} M, tadpoles and various species of fish become paralyzed after several hours exposure to the compound. It has been observed that frogs can tolerate very large doses of the compound (up to 10.0 mg/kg) without any apparent adverse effect.

SUMMARY

Compounds of the hemicholinium class have been demonstrated to produce neuromuscular blockade as well as inhibition at various sites in the autonomic nervous system. All experiments indicate that these compounds are acting on either preganglionic or postganglionic nerve terminals, and that inhibition of transmission is by a different mechanism than reported for other types of autonomic agents. The mechanism of action for these compounds is not known, and further studies on the biochemical level must be completed. The compounds do not appear to be particularly selective in their site of action, and further synthetic work will be required to produce compounds that will be more selective in their site of action.

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Pharmacology of 3,6 bis (3-diethylaminopropoxy) pyridazine bis-methiodide, a hemicholinium-like agent

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THE PRECEDING PAPERS of this symposium have dealt with the unusual pharmacologic properties of HC-3 and related hemicholiniums at various neuro-effector sites. Another compound, a structurally dissimilar bisquaternary pyridazine, 3,6 bis (3-diethylaminopropoxy) pyridazine bismethiodide (Win 4981) has been shown to share some of the effects of HC-3, at least at the neuromuscular junction.

The initial studies on Win 4981 emphasized the peculiar pattern of neuromuscular blockade produced, and the pharmacologic differences between this agent and *d*-tubocurarine and decamethonium (8, 9). Although no obvious explanation for these unique properties of Win 4981 could be offered at the time, a prejunctional site of action was not considered likely in view of the demonstrable postsynaptic effects of this compound. It was not until the studies by Long and coworkers (14, 22, 23) showing some striking resemblances between Win 4981 and HC-3, and the studies by MacIntosh *et al.* (1, 16–18) strongly suggesting a presynaptic site of action for HC-3, that this investigation of Win 4981 was extended to test this possibility with respect to its actions (10). These additional studies revealed other effects which these two compounds apparently have in common.

In this review of the pharmacology of Win 4981, its properties will be compared or contrasted to those of HC-3, *d*-tubocurarine, and decamethonium wherever possible; these compounds serving as a frame of reference. It is recognized that in so doing, there will be some repetition of certain areas specifically covered by other participants in this symposium.

NEUROMUSCULAR BLOCKING PROPERTIES

Activity of some bis-quaternary pyridazines. Win 4981 was originally selected from a large series of bis-quaternary pyridazines prepared by Steck *et al.* (28), which were screened for neuromuscular blocking activity (8). For this purpose the mouse inclined screen procedure and the cat sciatic-gastrocnemius preparation were used. The activity of the various members of this series, in which the number of methylene groups separating the pyridazine nucleus from the onium N centers and the quaternary ammonium substituents were varied systematically, is given in Table 1.

The most potent compound in the mouse, Win 4981, exhibited little or no blocking activity in the cat nerve-muscle preparation at doses up to 2 mg/kg. Higher doses were not tolerated, causing delayed deaths which appeared to be respiratory in nature. This observation prompted the more extensive evaluation of Win 4981 (described in the next sections) to determine if it did produce neuromuscular blockade as suggested by the mouse data. With reference to Table 1, it should be pointed out that the all-methyl compound in the propoxy series (Comp. IV) resembled decamethonium in its neuromuscular blocking action, whereas compound X with the aryl substituent on the quaternary nitrogens appeared to be curare-like. The all-ethyl compound (Comp. VI) was subsequently demonstrated to be similar to Win 4981, but less active. The di-methyl, ethyl quaternary ammonium analogue of Win 4981, was not prepared.

Influence of frequency of nerve stimulation. In view of the failure to reveal neuromuscular blocking activity of Win 4981 in the cat sciatic-gastrocnemius preparation, stimulated indirectly at the slow rate of 1/15 sec, the compound was retested in bilateral nerve-muscle preparations, in which the stimulation frequency of one side was increased to 1 per sec. Win 4981, over a wide range of dosage levels, was found to produce a characteristic slowly developing block of long duration on the side of the bilateral preparation stimulated at the faster frequency. These same dose levels were without effect on the contralateral nerve-muscle preparation stimulated at the 1/15 sec frequency (8). This unusual pattern of neuromuscular blocking action had not been previously described, although HC-3 was later demonstrated to produce similar effects in nerve-muscle preparations of various species (14, 22).

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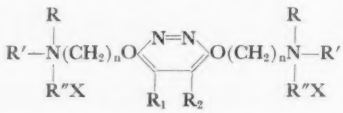
TABLE 1. *Neuromuscular Blocking Activity of Bis-Quaternary Pyridazines in the Mouse and in the Cat Nerve-Muscle Preparation (Modified from Gesler and Hoppe (8))*



Compound No.	n	R	R′	R″X	R_1	R_2	Mouse		Cat Nerve-Muscle Stim. Freq., 1 /15 sec	
							s.c. ED_{50} ± s.e. (mg/kg)	i.v. LD_{50} ± s.e. (mg/kg)	AED_{50} (mg/kg)	Duration (min)
I	2	Me	Me	MeI	H	H	16.0±1.0	28.0±1.4	>10.0	
II	2	Et	Et	MeI	H	H	3.3±0.3	5.9±0.5	6.6	15
III	2	Et	Et	MeBr	H	Me	0.8+0.1	1.8±0.16	2.8	8
IV	3	Me	Me	MeBr	H	H	0.6±0.1	1.0±0.2	0.046	15
Win 4981	3	Et	Et	MeI	H	H	0.15±0.01	0.63±0.05	>2.0	
VI	3	Et	Et	EtI	H	H	0.73±0.07	1.28±0.11	>1.0	
VII	3	Me	Me	NBBr*	H	H	0.3±0.05	0.47±0.02	0.089	5
VIII	3	Me	Me	NBBr*	H	Me	0.47±0.03		0.073	10
IX	3	Me	Me	NBBr*	Me	Me	0.46±0.04		0.162	5
X	3	Et	Et	NBBr*	H	Me	0.26±0.02	0.3±0.01	0.036	10

* 4-nitrobenzyl bromide.

The relationship between initial dose of Win 4981 and percentage of neuromuscular blockade, as determined in a total of 24 such preparations, is presented in Fig. 1. Comparative data for *d*-tubocurarine and decamethonium are also shown. It is significant that the dose-response relationship for Win 4981 and for HC-3 (14, 22) appears to be similar, but is of a different character than that for either of the reference agents. Even higher dose levels of Win 4981 (up to 2 mg/kg) did not produce complete neuromuscular blockade at the 1 per sec frequency of stimulation.

The median effective neuromuscular blocking doses (ED_{50}) of Win 4981, *d*-tubocurarine, and decamethonium, as determined at each of the two frequencies of stimulation, are compared in Table 2. It is evident that the frequency of nerve stimulation markedly influences the neuromuscular blocking potency of Win 4981. The paralyzing potency of *d*-tubocurarine was increased significantly, whereas that for decamethonium was unchanged by increasing the frequency of stimulation. The observation with respect to *d*-tubocurarine is in general agreement with the earlier reported findings of Preston and Van Maanen (21). Also, Foldes *et al.* (7) have recently reported that recovery of grip strength in human volunteers was dependent upon rate of exercise when partial neuromuscular block was produced by *d*-tubocurarine, but not when produced by depolarizing relaxants.

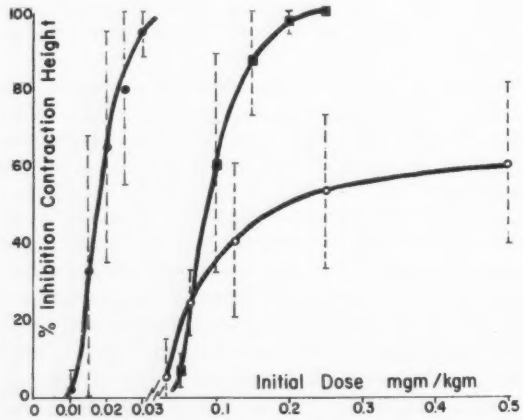


FIG. 1. Dose-response curves for decamethonium (●), *d*-tubocurarine (■), and Win 4981 (○), in the cat sciatic-gastrocnemius preparation stimulated at a frequency of 1 per sec. Mean responses ± 1 standard deviation are indicated for each dose. (From Gesler and Hoppe, 9.)

That the neuromuscular blocking potency of Win 4981 is dependent upon frequency of nerve stimulation is perhaps better demonstrated in experiments of the type shown in Fig. 2. Initially, both sides of a bilateral preparation were stimulated indirectly at a frequency of 3 per sec. After contraction responses had stabilized, stimulation of one side of the preparation was interrupted, allowing this nerve-muscle preparation to rest.

TABLE 2. *Influence of Frequency of Indirect Stimulation on Neuromuscular Blocking Potency of Win 4981, d-Tubocurarine and Decamethonium*

Compound	ED_{50} ± s.e. (mg/kg)	
	Stim. freq. 1/15 sec	Stim. freq. 1/sec
Win 4981	>2.0	0.190*
d-Tubocurarine	0.184±0.025	0.092±0.014
Decamethonium	0.018±0.002	0.018±0.002

* Approximate value.

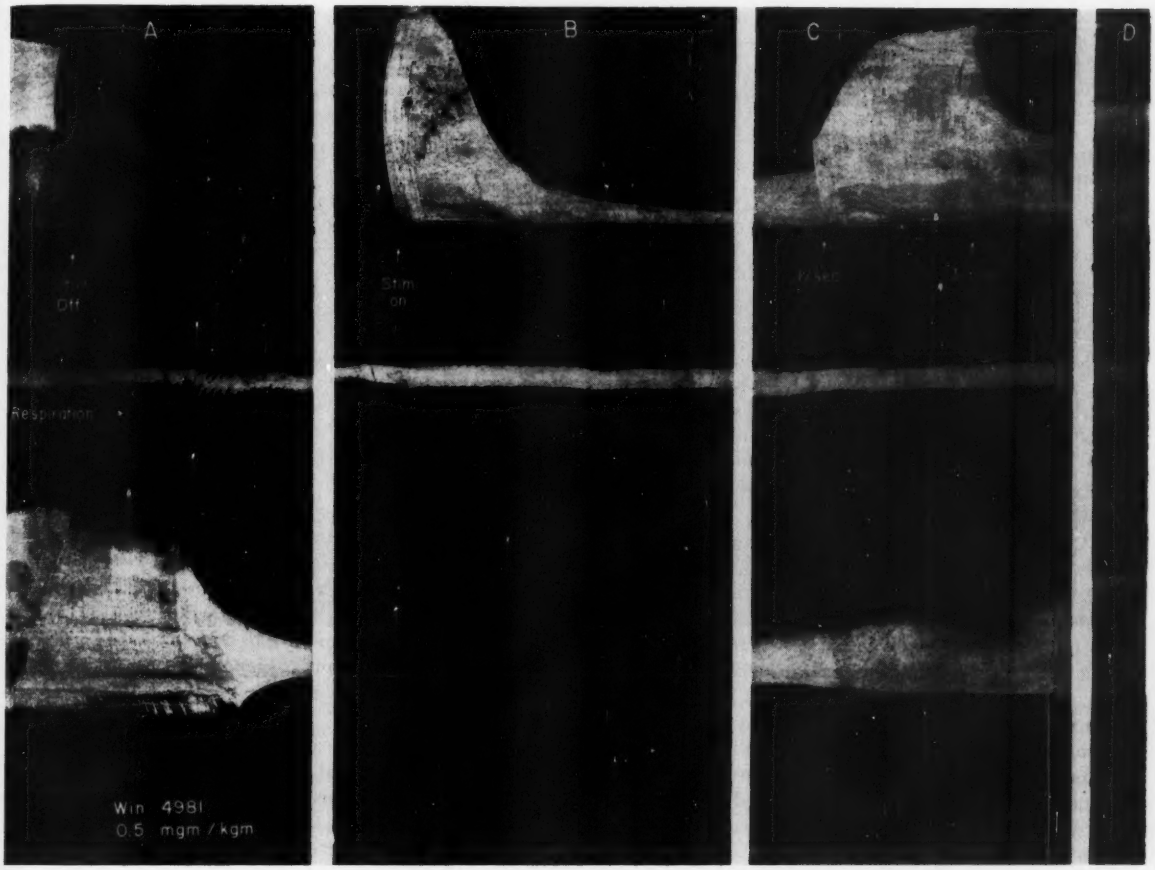


FIG. 2. Bilateral sciatic-gastrocnemius preparation. Both muscles stimulated initially at 3 per sec. Time in minutes. *B*, 70 min after *A*; *C*, 75 min after *B*; and *D*, 30 min after *C*. See text for explanation. (From Gesler and Hoppe, 9.)

Win 4981 was then injected intravenously, causing a gradually developing blockade of the continuously stimulated side of the preparation, which became complete after some 80 min. Stimulation of the rested contralateral nerve-muscle preparation was then resumed at a frequency of 3 per sec. It is evident that, initially, this rested muscle was not paralyzed, but required some 5–10 min of continuous stimulation before paralysis appeared. Beginning reversal of the blockade occurred in both preparations about 3–4 hr after the injection of Win 4981. As shown in Fig. 2 *C*, recovery was markedly accelerated in one of the muscles by reducing the frequency of stimulation to 1 per sec for a 30-minute test period. In very similar studies on HC-3, Reitzel and Long have observed, essentially, the same results (22).

These findings, in addition to demonstrating the influence of frequency of stimulation on the potency of Win 4981, also provide indirect evidence that the slow onset of neuromuscular blockade is neither the result of delayed access of Win 4981 to its site of action nor to the formation of an active metabolite. It was also established that Win 4981 has no important effects on either nerve or muscle, thus eliminating these structures as possible sites of action (9). Hence, this atypical pattern of neuromuscular blockade produced by Win 4981, as with HC-3, must result from some slowly developing alteration of normal transmission across the myoneural junction. This effect is not only dose dependent but, more importantly, is also dependent upon the rate of muscle stimulation through its nerve.

Following lethal intravenous dose levels or intra-arterial doses of 100 μg/kg or greater, Win 4981 produces a biphasic neuromuscular blockade (8, 10). The initial phase develops promptly, but is of short duration, and is followed by the characteristic, slowly developing sustained block. Such an atypical short-lived neuromuscular block has also been observed with HC-3 following lethal dose levels, even at a slow 1/10 sec frequency of indirect stimulation (22, 24).

Activity in unanesthetized animals. The slow onset and long duration of paralyzing effects, and the delayed deaths produced by Win 4981 in the 1 per sec nerve-muscle preparation, as with HC-3, are also evident in unanesthetized animals (8, 9). In contrast, the effects of

TABLE 3. *Effect of Level of Overt Activity in Mice on the Acute Toxicity of Win 4981 (From Gesler et al. (10))*

Pretreatment	Win 4981 i.v. LD_{50}* ± s.e. mg/kg	*d*-Tubocurarine i.v. LD_{50}* ± s.e. mg/kg
None (controls)	0.70±0.04	0.18±0.02
Amphetamine, 10 mg/kg i.p. 10 min before	0.43±0.06†	0.16±0.02
Chlormezanone, 400 mg/kg i.p. 30 min. before	1.75±0.09†	0.13±0.01‡

* Based upon deaths occurring within 60 min. † P = <0.01. ‡ P = 0.05.

d-tubocurarine and decamethonium are immediate and short-lived, or terminate quickly in death in both the unanesthetized animal and in the nerve-muscle preparation. In the pigeon, Win 4981 produces a flaccid, *d*-tubocurarine-like paralysis (9). To further differentiate Win 4981 from decamethonium, its paralyzing potency is subject to little species variability, at least in the cat, rabbit, pigeon, and mouse (8, 9).

As Giovinco has found with HC-3 (11), the acute toxicity of Win 4981 in mice is markedly influenced by the level of overt activity (Table 3). Animals rendered hyperactive by amphetamine pretreatment were significantly more susceptible to Win 4981, whereas mice made hypoactive by chlormezanone pretreatment were significantly less susceptible. The acute toxicity of *d*-tubocurarine, however, was not affected by amphetamine pretreatment, but was significantly "increased" in chlormezanone pretreated animals. These results are not inconsistent with the demonstrated influence of frequency of nerve stimulation on the paralyzing activity of Win 4981.

Antagonism studies. Neuromuscular blockade produced by Win 4981 in the cat sciatic-gastrocnemius preparation, stimulated at the 1 per sec frequency, is only partially reversed by neostigmine, and even less effectively by edrophonium (9, 10). In the unanesthetized rabbit, neostigmine pretreatment significantly increased the median head drop dose, but had no effect on the median lethal dose of Win 4981. Basically similar results were also obtained in the mouse (9). Reitzel and Long have more completely analyzed the neostigmine antagonism of HC-3 (22). While their findings may have no relevance to the neostigmine antagonism of Win 4981, it is interesting that the relatively short-lived block, demonstrable in their 1/10 sec nerve-muscle preparation following large doses of HC-3, was found to be more effectively antagonized by neostigmine than was the block revealed by a faster rate of stimulation at lower dose levels of HC-3.

Win 4981 also has anti-curare effects in the nerve-muscle preparation, particularly when stimulated at the slow 1/15 sec frequency. In preparations stimulated at the faster frequency this effect is of short duration and it is followed by the characteristic Win 4981 block. The fact that the interaction effects of Win 4981 and *d*-tubocurarine in the unanesthetized rabbit, whether paralysis or death is the end point, are additive rather than antagonistic (9), again illustrates that the actions of this agent in the intact animal are better correlated with its activity in the 1 per sec nerve-muscle preparation than in the preparation stimulated at the slower rate.

Whereas the anticholinesterase or anti-curare agents, or both, only partially reverse the paralyzing effects of Win 4981, choline chloride was found to be a considerably more effective antagonist. These special studies are considered separately in the section under Presynaptic effects.

SITE AND MODE OF ACTION STUDIES

Postsynaptic effects. A number of experiments have been carried out to delineate better those actions of Win 4981 referrable to the postjunctional neuromuscular transmission process (9). Not all of these actions of Win 4981 are shared by HC-3. For example, Win 4981 produces a contracture of the isolated frog rectus abdominis, but is only about 1/15th as active as decamethonium. HC-3 is apparently devoid of these excitatory effects. Neither Win 4981 nor HC-3 (22) inhibit the response of the rectus abdominis to added acetylcholine. However, Win 4981 at higher bath concentrations does potentiate acetylcholine. Based upon comparative studies with neostigmine, this effect of Win 4981 is probably attributable to its weak anticholinesterase activity (8, 9).

The response of the cat gastrocnemius muscle to close intra-arterially injected acetylcholine is reduced or abolished by intravenous pretreatment with Win 4981 at doses of 0.125 to 1.0 mg/kg. Brooks and Thies (4, see 26) have observed a similar effect of HC-3 in isolated nerve-muscle preparations.

Win 4981 also exhibits minimal excitatory effects in the denervated gastrocnemius of the cat, but it could not be differentiated from *d*-tubocurarine in this respect (9). Data on HC-3 in experiments of this type are not available.

Other actions of Win 4981, presumably related to postjunctional effects, can be briefly summarized (9). Relatively high doses of Win 4981 ($\geq$0.5 mg/kg) produce decamethonium-like fasciculations in the anesthetized cat. Such an effect has not been reported for HC-3. These fasciculations were not observed at lower dose levels, and neuromuscular blockade in the 1 per sec preparation was not preceded by a potentiation of the twitch height. The Win 4981 partially paralyzed cat gastrocnemius, like the curarized muscle, cannot sustain an indirectly delivered tetanus. However, Win 4981 differs from *d*-tubocurarine in that the tetanus is followed by only a slight posttetanic potentiation, which in turn is frequently followed by a greater degree of neuromuscular blockade. Desmedt (6) has observed similar results with HC-3 in a more detailed study of these phenomena.

These various observations indicate that Win 4981 does have the ability to alter certain of the sequential steps involved in the postjunctional transmission of a

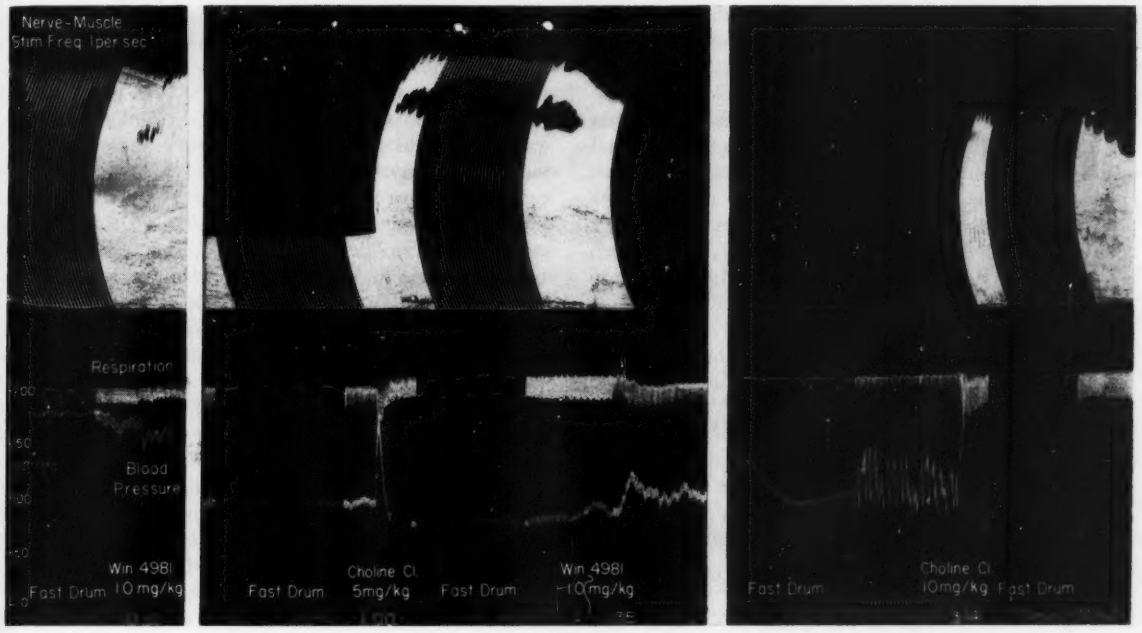


FIG. 3. Effects of choline chloride in reversing the neuromuscular blocking action and the respiratory paralyzing action of Win 4981 in the cat. "Fast drum" indicates brief periods when the kymograph speed was increased about 50-fold to characterize the respiratory excursions. First panel, preinjection record. Middle panel, about 2.5 hr after Win 4981 with beginning recovery from neuromuscular blockade and labored inspiration (downstroke). Complete, though temporary, reversal of both effects follows choline chloride. Last panel, about 1.5 hr after second Win 4981 injection with 100% nerve-muscle blockade and irregular, extremely labored respiration. Note the almost complete and immediate reversal of both effects following the 10 mg/kg dose of choline. (From Gesler *et al.*, 10.)

nerve impulse. It is also apparent that certain differences do exist between Win 4981 and HC-3 in their postsynaptic effects. The significance of these findings, and their relevance in providing an explanation for the unusual paralyzing activity of Win 4981, cannot be as easily surmised. In this regard, probably the most meaningful of the various postsynaptic effects of Win 4981 is the ability to antagonize the twitch response to close intra-arterially injected acetylcholine. Such an effect of Win 4981 could be shown at intravenous dose levels which would be expected to produce severe paralysis in the intact nerve-muscle preparation or in the unanesthetized animal. Although this might suggest a direct "cause and effect" relationship, the association is not this clear-cut; for Brown *et al.* (5) have demonstrated that curare is considerably more active in reducing the muscle twitch response to close intra-arterial acetylcholine, than in blocking the indirectly stimulated muscle.

A consideration of the postsynaptic effects of HC-3 has occupied a large portion of the discussion in this symposium (Brooks and Thies (4); Martin and Orkand (20); see also (26)). These investigators have suggested that the neuromuscular blockade produced by HC-3 is the result of two distinct processes, in which a postsynaptic action becomes important at relatively high dose levels. This interpretation of a dual site of action for HC-3 appears to be equally applicable to Win 4981. It would thus seems reasonable to conclude, at least tentatively, that these demonstrable postsynaptic effects of Win 4981 are probably unimportant in its primary paralyzing action, but may be of secondary importance at toxic dose levels.

Presynaptic effects. Schueler first observed that the toxic effects of HC-3 in mice could be effectively antagonized by choline chloride (25). This observation was later pursued by Long and co-workers, and was the basis for a series of interesting papers, by this group, in which this phenomenon was more completely assessed (14, 22, 23). The experimental findings relative to the rather selective choline antagonism of HC-3 are comprehensively reviewed by Long in the preceding paper (15). MacIntosh independently reported that HC-3 depressed acetylcholine synthesis in intact neurones (16–18). These results strongly implicate a presynaptic site of action for HC-3. Very similar but less complete data have been obtained on Win 4981, suggesting that both of these agents act via a common site and mode of action.

Choline chloride effectively antagonizes not only HC-3, but also Win 4981. This has been shown in the mouse, rabbit, and cat (10). The immediate effectiveness of choline chloride in reversing the neuromuscular blockade produced by a "toxic" dose of Win 4981 in the cat sciatic gastrocnemius preparation is demon-

strated in Fig. 3. The lower portion of the tracing illustrates also the simultaneous choline reversal of the obviously labored respiration produced by Win 4981 in this same animal.

Since choline effectively and dramatically restores spontaneous respiratory activity to an essentially normal level in such Win 4981 poisoned animals, and since this effect mimics its action in the isolated nerve-muscle preparation, there seems to be little reason to suspect a central component of action for Win 4981, as has been postulated for HC-3 (12, 13, 25, see 2). In this connection Schueler's initial observation (25) that HC-3 causes a simultaneous depression of the knee jerk reflex and respiration in the anesthetized rabbit, without affecting the isolated sciatic-gastrocnemius preparation stimulated at a 1/10 sec frequency, is of interest. Since the influence of frequency of stimulation on the neuromuscular blocking potency of HC-3 was not recognized at that time, these results were indeed indicative of a central action. However, if the primary site of action of HC-3 is in fact peripheral, then the inhibitory effects of HC-3 on the knee jerk reflex must also be explicable on a peripheral basis. This question has apparently not been investigated with respect to HC-3. However, some experiments carried out on Win 4981 are pertinent (Gesler, unpublished observations). In the high spinal (C_2) or chloralosed cat, Win 4981 at intravenous dose levels of 0.100–0.5 mg/kg produced a 50–100% (AED_{50}[2] = 0.100 mg/kg) slowly developing block of the isotonically recorded knee jerk reflex elicited automatically at 10-sec intervals, using the solenoid tapper described by Schweitzer and Wright (27). These same doses had no clear-cut effect on the isolated nerve-muscle component (femoral nerve-quadriceps muscle preparation) of this reflex arc in the contralateral leg, which was stimulated indirectly at a 1/15 sec frequency. Thus, Win 4981 inhibits the knee jerk reflex as does HC-3, without inhibiting the peripheral effector component of the reflex. *D*-tubocurarine, on the other hand, was found to produce a prompt, short-lived block of the knee jerk reflex (AED_{50} = 0.125 mg/kg), and was essentially equi-effective on the contralateral femoral nerve-quadriceps muscle preparation. Although these findings would suggest a central site of action for Win 4981 as they did for HC-3, such a conclusion soon became untenable. Additional studies established that this blocking action of Win 4981 was dependent upon its gaining access to the peripheral components rather than the central connections of the reflex arc, since the intra-aorta injection of 25–50 μg/kg of Win 4981 blocked only the arterially nonoccluded side of bilateral knee jerk preparations. The results of these experiments do not exclude the peripheral afferent elements of the reflex as a possible site of action; but in view of the known effects of Win 4981 at the myoneural junction, and particularly in view of the influence of frequency of stimulation on its neuromuscular blocking potency, it seems more likely that this action on the knee jerk reflex is related to the high frequency motor discharge rate of stretch reflexes.

Returning to the choline reversal of Win 4981 induced blockade, the specificity of this antagonism was investigated in several ways (10). A series of choline analogues were prepared and tested in the Win 4981 partially paralyzed nerve-muscle preparation. Only those compounds presumed to be precursors of acetylcholine or acetylcholine-like substances (dimethylethylaminoethanol iodide and dimethylaminoethanol in addition to choline) were found to show any sustained antagonistic action. Methoxy choline, which of course cannot serve as an acetylcholine precursor, was included in this group of choline analogues as a crucial test of this hypothesis. Although it produces a short-lived reversal of the Win 4981 blockade, probably the result of its high cholinergic activity, it does not have a sustained antagonistic effect even at just sub-lethal dose levels. The triethyl analogue of choline was found to potentiate the Win 4981 block. Interestingly, Bowman and Rand (3) have recently reported that this choline analogue is also hemicholinium-like in its neuromuscular blocking action.

To obtain other definitive but admittedly indirect evidence as to whether choline exerts its action via a presynaptic or postsynaptic mechanism, the effectiveness of choline chloride in reversing paralysis caused by both *d*-tubocurarine and Win 4981 was compared with that of edrophonium (10). It was found that edrophonium promptly restores the curarized muscle to normal, but has an unimpressive effect on the Win 4981 paralyzed muscle; whereas choline is a more specific antagonist of Win 4981 than of *d*-tubocurarine. Similarly, in the mouse, choline pretreatment significantly antagonizes both the paralyzing and lethal effects of Win 4981, but not of *d*-tubocurarine. The results of these experiments exclude, or at least minimize, the excitatory (cholinergic) action of choline as being an important causal factor in its effectiveness to normalize the Win 4981 poisoned muscle.

Another interesting observation with respect to the choline reversal of Win 4981 induced neuromuscular blockade is the selective antagonistic action of choline demonstrable in the biphasic block. It was noted in the first section that following large intravenous dose levels, or upon intra-arterial injections of Win 4981, an immediate but transient neuromuscular blockade precedes the more slowly developing and sustained blockade characteristic of this compound. Choline chloride exerts little, if any, antagonistic action on the initial phase, but acts only to reverse or prevent the slowly developing phase of such a biphasic block (10). Thus, it is possible to distinguish pharmacologically between these two phases of the neuromuscular blockade produced by relatively large doses of Win 4981. It was previously suggested that the initial, short-lived phase of the biphasic block produced by Win 4981 may result from its weak postsynaptic effects. This phase of the block is not choline reversible; and, on the basis of the weak

[2] Approximate median paralyzing dose.

excitatory effect of Win 4981, is probably decamethonium-like in nature.

On the other hand, in view of the vast amount of circumstantial evidence available, the more important blocking action of Win 4981, characterized by a slow onset and long duration, appears to result from a prejunctional mechanism, as has been suggested for HC-3. Unfortunately, the evidence for a presynaptic site of action for Win 4981 is not as complete or as direct as is the case with HC-3. Farah (personal communication) has found that Win 4981, like HC-3, strongly inhibits both *N*-methylnicotinamide and tetraethylammonium ion transport in dog renal slices. Since choline is transported by the same mechanism which transports NMN and TEA, these findings would suggest a rather specific presynaptic mechanism common to both agents. Furthermore, the many other described similarities between Win 4981 and HC-3, in effects at the myoneural junction, suggest a common site and mode of action. While this working concept is attractive, since it does provide a plausible explanation for the unusual paralyzing properties of Win 4981, it is presently supported by only indirect evidence. Further studies designed to test directly this hypothesis are clearly needed.

OTHER EFFECTS

HC-3 has also been extensively studied for effects at other neuroeffector sites (15, 19; see also 26). Such a systematic investigation of Win 4981 at these other structures has not been undertaken, but would be of extreme interest in view of the striking resemblance in the effects of these two agents at the neuromuscular junction. Only very gross descriptive data are presenty available on Win 4981. It apparently has minimal cardiovascular effects, in the anesthetized cat, at dose levels up to about 0.4 mg/kg. Higher dose levels produced an inconsistent hypotensive response (8). These descriptive findings certainly contribute little to an intimate understanding of the actions of Win 4981, if any, at the cholinergic sites involved, but may be suggestive that such effects do occur.

SUMMARY

Win 4981 has a number of unusual properties at the neuromuscular junction, which very closely resemble those of the hemicholinium, HC-3, but which serve to differentiate it from either *d*-tubocurarine or decamethonium. The most striking of these is the pattern of neuromuscular blockade in the anesthetized cat. Paralysis is dependent upon the frequency of nerve stimulation and is characterized by a slow onset and a relatively long duration of action. At lethal dose levels, deaths are delayed and appear to be the result of peripheral respiratory failure. This same pattern of action is also seen in the unanesthetized animal. Anticholinesterases or anti-curare agents only partially antagonize the paralyzing effects of Win 4981, but choline chloride is almost a specific antagonist.

Although Win 4981 has some minimal excitatory effects, which must be associated with a postsynaptic site of action, these appear to be relatively unimportant insofar as its principal neuromuscular blocking action is concerned. On the basis of the experimentally demonstrated specificity of the choline antagonism of Win 4981, and the similarities between this agent and HC-3, it is postulated that Win 4981, like HC-3, produces neuromuscular blockade primarily via a presynaptic mechanism.

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Respiratory depressant effect of HC-3[1]

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When SCHUELER (13, 14) first described the delayed respiratory paralysis resulting from small doses of hemicholinium (HC-3), he was well aware that larger doses, of the order of 5–10 times the minimum requirement, caused early neuromuscular blockade. The delayed paralysis, not attributable to interference with neuromuscular transmission, was therefore considered to be the "primary" action of the drug. Indeed, failure of respiration, after HC-3, commonly results in violent asphyxial convulsions. Schueler further contended that this late effect was mediated centrally through depression of spinal relay centers for respiration. Kasé and Borison (7) studied the respiratory paralyzant effect of HC-3 by means of brain stem stimulation and transection. We agreed with Schueler that the primary depressant action of the smallest effective doses of HC-3 is central in nature, but we concluded that this action is effected at the brain stem rather than the spinal level. In addition, we also observed neuromuscular blockade produced by HC-3, and we too attributed this peripheral effect to a "secondary" action manifested after doses larger than required for the delayed central effect. Other workers (6, 9), however, would deny altogether that a central action of the drug participates in depression of the respiration.

CENTRAL, PERIPHERAL OR BOTH?

A peripheral action may explain one kind of respiratory paralysis due to HC-3, but such an explanation neither excludes nor even militates against a central action. As a case in point, Holmes and Wilson (6) recorded, in cats, high intensity inspiratory discharge from the medulla oblongata at a time when the diaphragmatic electrogram fell silent after intravenous HC-3. However, they found it necessary to employ doses of 3–4 mg/kg and they obtained a paralyzant effect within 30 min. According to our experience, this result is to be expected from such a comparatively large dose of HC-3 and after such a relatively short interval of observation. Curiously, Holmes and Wilson reported that respiratory activity was affected little by the dose of 2 mg/kg. This discrepancy in dosage requirement, compared with results of others, could be explained by insufficient time allowed for the onset of central effect. By contrast, Longo (9) found that respiratory paralysis resulted in cats and rabbits after a latency of 1–2 hr following an intravenous dose of 0.5 mg/kg, and he observed concurrently an augmentation in phrenic discharge. Characteristically, after minimal doses of HC-3, the respiration passes through a phase of augmented irregular gasping. Longo unfortunately recorded ventilatory movement, in a single plane, by means of a thread tied to the skin of the upper abdomen; it is possible that the gasping phase was not mechanically registered while phrenic discharge was enhanced.

EVIDENCE FOR A CENTRAL ACTION

Now, let us examine the available evidence for a delayed central respiratory paralyzant action of HC-3, keeping in mind that the neuromuscular blocking action serves to complicate the picture.

Fig. 1 and 2 are taken from the work of Kasé and Borison (7). We stimulated the medulla oblongata electrically with the aid of a stereotaxic instrument, and recorded respiration pneumographically. In Fig. 1 are shown the inspiratory and vasodepressor responses to stimulation of the medial reticular formation with threshold, submaximal, and maximal currents in a pentobarbitalized cat.

RESPONSES FROM MEDULLA OBLONGATA

The following points deserve emphasis: *1*) As a consequence of 1 mg/kg intravenous injection of HC-3, ongoing respiration became gasp-like and irregular, and terminated after 2 hr. *2*) Threshold voltage requirement for sustained inspiration became progressively greater, yet the maximal response remained essentially unaltered. *3*) Some improvement in central excitability was evident at 170 min at the time that spontaneous breathing had temporarily recovered. *4*) Resting blood pressure, as well as vasodepressor reactivity to stimulation, diminished *pari passu* with failure of the respiration. The elevated blood pressure at 120 min can be ascribed to hypoxia resulting from inadequate ventilation.

Thus, the conclusion is permissible that the final common pathway for the act of breathing was functional at a time when ventilatory cycling activity had ceased.

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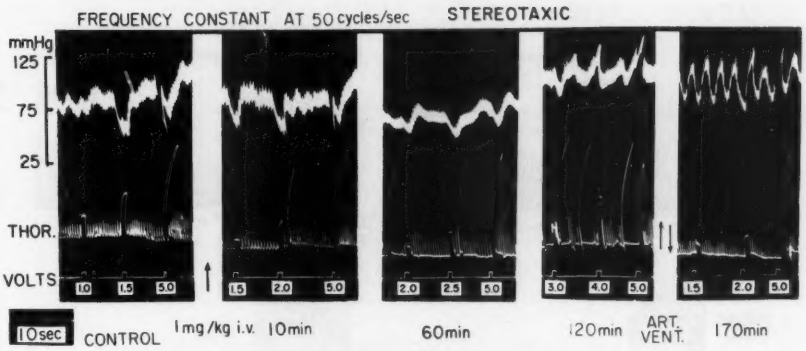

FIG. 1. Effect of 1.0 mg/kg i.v. HC-3 on threshold, submaximal and maximal stimulus requirement for respiratory and vasomotor responses to bipolar electrical stimulation of the medial reticular formation in the medulla oblongata of a cat anesthetized with pentobarbital Na. Respiration recorded via thoracic pneumograph, inspiration up. Artificial ventilation administered between 120 and 170 min after HC-3 injection. Stimulus pulse duration constant at 1 msec in this and subsequent experiments.

This effect we attribute to selective depression by HC-3 of the medullary respiratory oscillator. To be stressed is the fact that medullary interneurons, as well as primary and secondary relay neurons, intervene between our point of stimulation in the brain stem and the effector muscles. It seems unreasonable to invoke an explanation of unphysiologic breakthrough at the neuromuscular junction when the central stimulus must traverse such a complex neuronal path to the respiratory musculature. In fact, similar effects on respiration and blood pressure, as shown in Fig. 1, were produced by intracarotid injection of as little as 0.1 mg/kg of HC-3, which is an ineffective dose by the intravenous route.

The alteration in pattern from a eupneic to a gasping form of breathing after HC-3—that occurs considerably in advance of respiratory paralysis—can best be explained as resulting from an inhibition of supramedullary modulating influence on the respiratory integrator. This is consistent with the effects of other drugs as, for example, sodium cyanide, and of certain surgical procedures on the brain stem (2). Ability of the respiratory muscles to hold the inspiratory position during the gasping phase is demonstrated by the sustained response to medullary stimulation.

Finally, the fall in blood pressure and reduction in central vasomotor reactivity, observed following HC-3, further supports the notion of a depressant action of the drug on the brain stem reticular formation.

COUGH REFLEX

Perhaps our most compelling argument for a central action of HC-3 is the observation that repetitive cough, both reflexly and centrally evoked, remained undiminished, if not increased, in intensity after respiratory arrest had resulted from adequate doses of the drug. Elicitation of such vigorous activity is surely not compatible with the presence of neuromuscular paralysis.

As an outcome of studies on the influence of various drugs, of electrical stimulation, and of surgical intervention on the central respiratory mechanism (2), we have characterized a pacemaker region in the medulla which is concerned in the coordination of specialized augmented repetitive respiratory activity. This region has been further subdivided into an expiratory pacemaker for multiple cough, and an inspiratory pacemaker for multiple gasp.

In Fig. 2 are shown thoracic and abdominal pneumographic tracings of repetitive gasping responses elicited by stimulation of the medullary inspiratory pacemaker in an unanesthetized, decerebrate, decerebellate, and vagotomized cat. Notice that at 55 min and again as late as 196 min after 2 mg/kg intravenous HC-3, when spontaneous breathing had ceased, the gasping response was elicited without decrement. Another observation of interest is that, during the temporary recovery of spontaneous breathing at 175 min, the inspiratory shift in the baseline seen during control stimulation again became manifest, thereby suggesting a transitory recovery of central excitatory state.

From this experiment, we may once more conclude that the final common pathway for the act of breathing

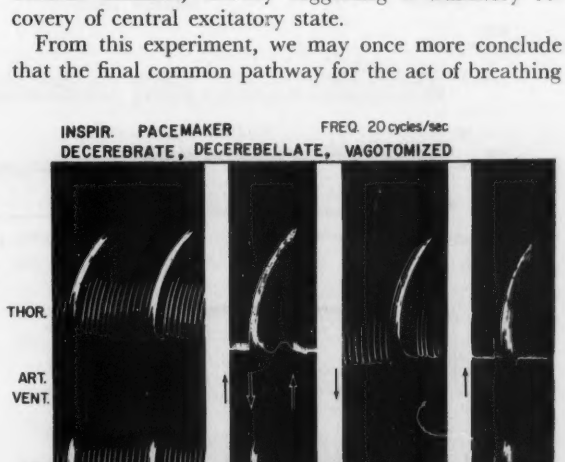

FIG. 2. Effect of 2.0 mg/kg i.v. HC-3 on repetitive gasping responses to bipolar electrical stimulation of inspiratory pacemaker in floor of fourth ventricle of an unanesthetized, decerebrate, decerebellate, and vagotomized cat. Artificial ventilation given when required as indicated by on-off arrows.

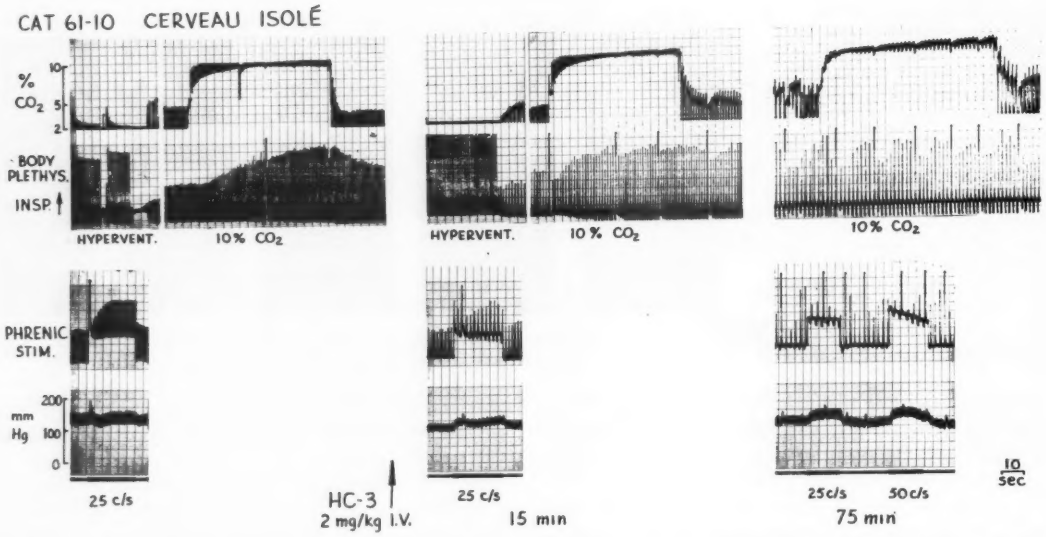

FIG. 3. Effect of 2.0 mg/kg. i.v. HC-3 on sensitivity to carbon dioxide and on response to stimulation of the phrenic nerve (in the neck) in an unanesthetized *cerveau isolé* cat.

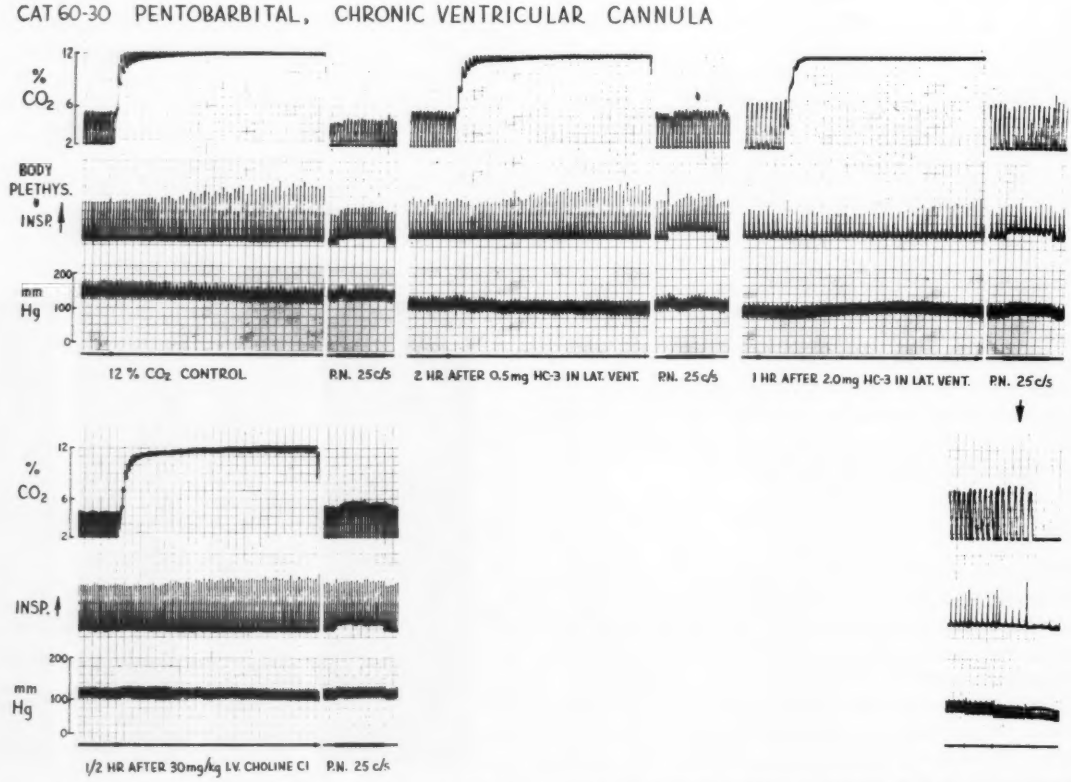

FIG. 4. Effect of intraventricular HC-3 on sensitivity to carbon dioxide, response to phrenic nerve stimulation, and resting blood pressure in a pentobarbitalized cat with chronic lateral ventricular cannula. P. N. refers to stimulation of phrenic nerve. Note counteracting effect of 30 mg/kg i.v. choline Cl. Choline administered after 0.5 mg/kg i.v. atropine so_4 in this and subsequent experiments.

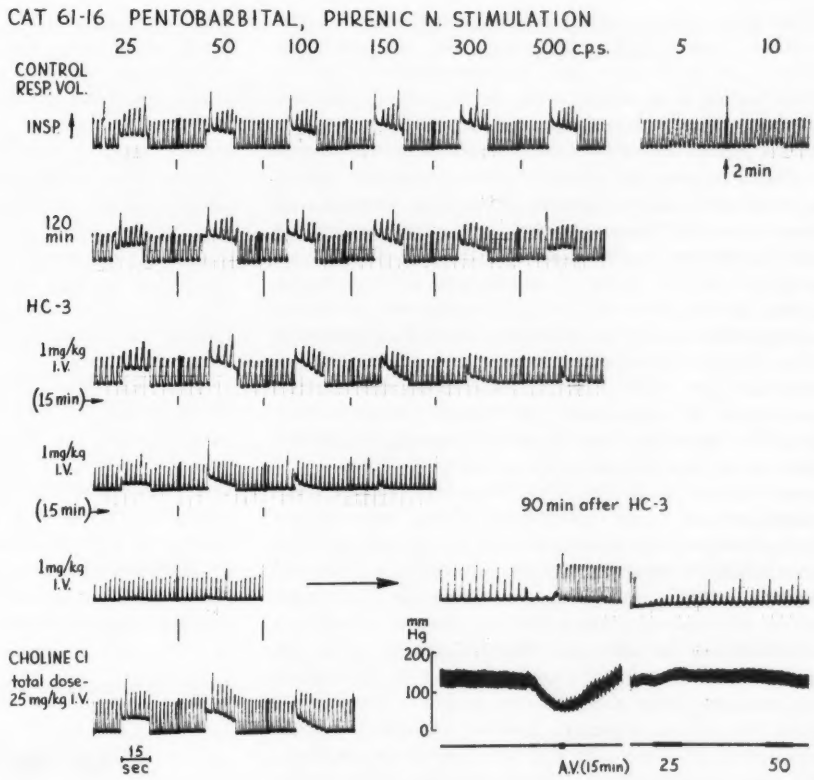

FIG. 5. Effect of i.v. HC-3 on frequency spectrum of diaphragmatic response to phrenic nerve stimulation in pentobarbitalized cat. Note counteracting effect of 25 mg/kg i.v. choline Cl.

remained functional after respiratory arrest due to HC-3. Moreover, the drug selectively spared the pacemaker mechanism for repetitive coughing and gasping.

SENSITIVITY TO CARBON DIOXIDE

In the past few months, we have again investigated the respiratory depressant action of HC-3, employing, however, electrographic recording of ventilation by means of a body plethysmograph, and of end-expiratory CO_2 by means of an infrared analyzer. Blood pressure was also recorded.

Fig. 3 shows the effect of 2 mg/kg intravenous injection of HC-3 in an unanesthetized, *cerveau isolé*, cat. The control records, on the left, show that *a*) apnea resulted after a brief period of mechanical hyperventilation which reduced the expired CO_2 level to 2%; *b*) administration of 10% CO_2 resulted in a marked hyperpnea; and *c*) effective stimulation of the phrenic nerve at 25 cycles per second caused a sustained shift in respiratory baseline. As early as 15 min following injection of HC-3, hyperventilation no longer induced apnea; the respiratory rate was diminished; the pattern of breathing became irregular, augmented, and gasp-like; 10% CO_2 was less effective in stimulating hyperpnea; and phrenic stimulation continued to elicit a sustained inspiratory shift.

At 75 min after drug administration, shortly before breathing ceased, the resting expiratory CO_2 level was elevated; breathing was considerably slower and still more irregular, but undiminished in amplitude; 10% CO_2 was entirely without effect; and, phrenic stimulation continued to be effective. Blood pressure fell slightly, but not sufficiently to be responsible for the effects observed on the respiration.

This new finding, of reduced sensitivity to CO_2 resulting from administration of HC-3, throws still another light on the mode of respiratory depressant action of the drug. At the same time, it further strengthens the contention that HC-3 affects the central respiratory apparatus.

INTRAVENTRICULAR INJECTION

When we reported our results in 1958 (7), we stated that cerebral intraventricular administration of HC-3 had no effect on respiration. This conclusion was based on a few acute experiments, in which cannulation of the lateral ventricle was attempted without prior experience. Schueler had previously reported that the dosage requirement of HC-3 for respiratory paralysis was greater by the intraventricular than by systemic routes of administration. In our latest series of experiments, after extensive experience with chronic cannulation of the lateral ventricle, we found on repeated trials with HC-3 in unanesthetized cats that the drug was more effective

when given intraventricularly than when given intravenously. Remarkably, the latency for the paralyzant effect was not shorter after intraventricular injection. This finding is in accord with the hypothesis that the blocking effect of HC-3 on synaptic transmission depends on the exhaustion of preformed stores of acetylcholine.

Fig. 4 shows the effect of intraventricular HC-3, administered via a chronic indwelling cannula, on respiration and blood pressure in a pentobarbitalized cat. Respiration was again recorded via a body plethysmograph. P. N. refers to stimulation of the phrenic nerve. At 2 hr after a total dose of 0.5 mg one can detect a somewhat diminished stimulant effect of administered CO_2. This is consonant with the rise in resting level of end-expiratory CO_2. Other points of interest are the slowing of the respiration, the tendency toward more gasp-like breathing, the unaltered response to phrenic stimulation, and the fall in blood pressure. When it was clear that the peak effect had been reached, an additional dose of 2 mg was injected. On a body weight basis, the total dose was equivalent to 0.75 mg/kg. One hour after the second dose, the sensitivity to CO_2 had essentially been abolished, the respiration had converted to a slow gasping form, but phrenic stimulation continued to be effective. Blood pressure again fell slightly but definitely. The lower panel on the right shows the terminal phase that followed within a few minutes after the above sequence. Choline chloride was administered intravenously and the record on the lower left taken one half hour after a total dose of 30 mg/kg shows the recovery, although perhaps not complete, in respiratory pattern, CO_2 sensitivity, and blood pressure level.

Thus, the same train of events can be initiated by intraventricular as by intravenous injection of HC-3, with dosage and latency consistent with a primary central action of the drug.

PHRENIC NERVE STIMULATION

The question naturally arises whether by direct phrenic nerve stimulation we are not causing unphysiologic breakthrough at the diaphragmatic neuromuscular junction.

Fig. 5 shows the frequency-response spectrum of phrenic nerve stimulation before and after administration of HC-3. On the top line are shown responses to stimulation at frequencies varying from 5–500 cycles/sec delivered for periods of 15 sec at 2-minute intervals. Frequencies below 25 cycles, shown in the upper right panel, were not effective. The response was only slightly enhanced at frequencies above 100 cycles, but with increasing fall-off. After 2 hr without treatment, responses up to 150 cycles were essentially unaltered, but some diminution in response is evident at the higher frequencies. It should be stressed at this time that, from information available in the literature (1, 8, 12), the normal discharge rate in the phrenic nerve is usually less than 50 cycles per sec and, under extreme asphyxial conditions, may reach as high as 100 cycles per sec. Rates of phrenic stimulation employed in our other experiments, that is, 25 and 50 cycles per sec, well approximate the physiological range of activity.

Following intravenous administration of HC-3 in 1 mg/kg doses at 15-min intervals, it is clear that the drug produced a prompt effect at the phrenic-diaphragm junction which manifested itself first and for the most part at the higher frequencies. Although the neuromuscular junctions in question appeared to be completely blocked in 45 min, the respiration did not fail until 90 min after drug injection. Choline restored both spontaneous breathing and phrenic response to frequencies below 100 cycles.

Thus, sustained response to phrenic stimulation can be relied upon to indicate that neuromuscular blockade does not exist, but a diminished response to stimulation does not necessarily reflect the degree of interference with physiological activity.

Finally, in Fig. 6 is shown the effect of a large dose (12 mg of HC-3), injected intraventricularly, sufficient to produce neuromuscular blockade if given systemically. At 30 min after injection, the response to phrenic nerve stimulation was all but obliterated, and sensitivity to

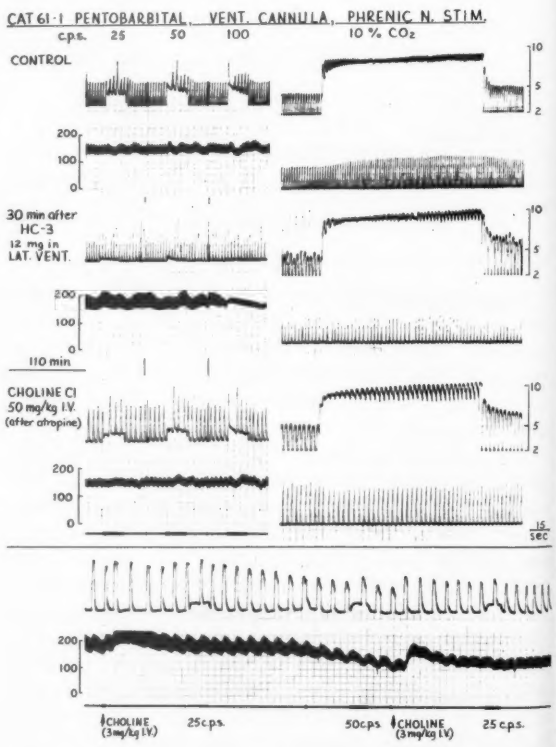

FIG. 6. Effect of large dose of intraventricular HC-3 (12 mg) on sensitivity to carbon dioxide, response to phrenic nerve stimulation, and resting blood pressure in pentobarbitalized cat with chronic lateral ventricular cannula. Note counteracting effect of 50 mg/kg i.v. choline Cl. Lowermost panel shows recovery sequence of respiration from gasping to apneustic to eupneic forms.

CO_2 was lost. However, spontaneous respiration of a gasping type persisted for approximately 1.5 hr longer. It is thus apparent that HC-3 rapidly escaped from the cerebrospinal fluid into the blood, but the latency for respiratory paralysis was not reduced, indicating that an insufficient amount of the drug had been released into the general circulation to cause complete neuromuscular blockade. Administration of choline, which incidentally was invariably preceded by atropine in all the experiments shown, only partially restored control conditions. In this experiment, of particular interest was the nature of recovery of the respiration, shown in the lowermost panel. This sequence in pattern from gasping to apneustic and, finally, to a more normal type of breathing argues strongly for recovery of the pontine respiratory modulator brought about by choline reversal of the HC-3 effect.

Is it, after all, surprising that HC-3 should have a central action? A cholinergic mechanism has long been postulated as playing a key role in central regulation of breathing (5, 10). The deleterious effect of anticholinesterases on respiration and the counteracting influence of atropine are well known. That these drugs act centrally to produce their effects is no longer doubted (3, 4, 11, 15–18). It is, therefore, not surprising that HC-3 is capable of producing respiratory paralysis through a central action on cholinergic transmission.

SUMMARY

In conclusion, I shall enumerate the various lines of support for a primary delayed central respiratory paralyzant action of HC-3. These arguments in no wise minimize the importance of neuromuscular blockade as a separate phenomenon. *1*) Unanesthetized animals have overt, motor convulsions when breathing stops following adequate doses of HC-3. *2*) Less HC-3 is needed by the intracarotid and cerebral intraventricular routes than by the intravenous route to produce respiratory arrest. *3*) Conversion of eupnea to gasping is evidence for pontine depression by HC-3. *4*) Threshold excitability of the inspiratory center is diminished before breathing has stopped. *5*) A maximal sustained response is elicitable from the inspiratory center after breathing has stopped. *6*) Undiminished cough responses are elicitable, both reflexly and centrally, after breathing has stopped. *7*) The respiratory center becomes insensitive to CO_2 before breathing has stopped. *8*) Stimulation of the phrenic nerve remains effective after breathing has stopped. *9*) Central vasomotor excitability is depressed simultaneously with depression of respiration.

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C. JUDSON HERRICK

It is to the memory of this man that the Symposium on the Neurophysiology of Learning and Behavior was dedicated.

NEUROPHYSIOLOGY OF LEARNING AND BEHAVIOR[1]

In Memoriam: C. JUDSON HERRICK (1868–1960)

PROGRAM

Introduction ..... ROBERT B. LIVINGSTON
Growth of neurophysiological concepts relating to learning and motivation .. CLIFFORD T. MORGAN
Neurophysiological studies on learning and motivation ..... ROBERT GALAMBOS
Some developments in brain lesion studies of learning ..... ROGER W. SPERRY
Brain mechanisms and the learning process ..... W. R. ADEY
Nervous mechanisms of behavior ..... (Read for C. Judson Herrick)

Introduction

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IN OCTOBER 1959, the Council of the American Physiological Society proposed holding a half-day symposium, at the time of the April 1960 meeting of the Society, on the subject of the Neurophysiological Basis of Learning and Motivation. This was to be the first symposium ventured under sponsorship of the Society which would be concerned with neurophysiology in its relations with psychology. Dr. Robert Galambos promptly made the felicitous suggestion that Professor C. Judson Herrick, who had in that same week just turned his 91st birthday, should be invited to be Chairman of the Symposium. Professor Herrick had contributed much of the groundwork of detailed neuroanatomy upon which brain physiology so obviously depends; moreover, he had contributed some of the most powerful functional generalizations as to what mechanisms must underlie learning and motivation and how these may be conceived to operate in terms of both structure and function, including the subjective as well as objective aspects of neuronal processes. Professor Herrick was invited to be Honorary Chairman. It was represented to him that the advantages to being Honorary Chairman are substantially greater than those of the ordinary Chairman: if he felt unable to attend, or did not wish to officiate at the meeting, he could still be Honorary Chairman. We hoped that he would be so interested in the subject matter and other participants in the Symposium that he would enjoy being Chairman *de facto* as well as Honorary.

Professor Herrick set about immediately to write a paper "primarily for the active participants of this conference." Within a short time he sent his manuscript, hammered out on his own typewriter. "Most of this matter is trite, but I feel that it is a good thing to be reminded in various contexts of familiar bits of common knowledge so that we may set the detail of a particular inquiry into its appropriate niche in the imposing structure of science as a whole." He spoke embarrassedly about the fact that he had to finish and submit his paper before he had been able to obtain copies of the new *Handbook of Physiology*, Section I, Volume 1, which had just been announced by the American Physiological Society, and the symposium on Brain Function and Learning which was shortly to take place under the auspices of the American Association for the Advancement of Science.

On January 29, 1960, Professor Herrick died at his home in Grand Rapids, Michigan. The symposium conceived in his honor had to be held in his memory. His last paper was read before the symposium audience and portraits of Professor Herrick provided for the occasion by his daughter, Dr. Ruth Herrick, were projected during this reading to add a more intimate touch from this pioneer in basic neurology and psychology.

[1] A symposium held in Chicago, Ill., April 12, 1960. Sponsored by the American Physiological Society.

"We know a man by his words and deeds. What a man does is his life. As a man thinketh in his heart, so is he; but thinking is doing, and when thoughts are uttered the words express more than ideas; they mirror the man because they express his inner nature, they are a part of him." (In: *George Ellett Coghill, Naturalist and Philosopher*. Chicago, Univ. of Chicago Press, 1949, p. 3.)

"[About 1890 Professor Henry Fairfield Osborn at Princeton] conceived the idea that it might be possible to . . . [cut] serial sections through the entire body of a small animal and then [analyze] each nerve trunk under the microscope into its components. If each component were traced peripherally to its end-organs and centrally to its proper station within the brain, then the brain itself would be analyzed into regions defined by their peripheral connections, sensory or motor as the case might be. Thus a physiological analysis would be effected by an anatomical method and morphology would begin to make sense in terms, not of abstract principles, but of actual use in the operations of adjustment.

"This research was undertaken by one of Professor Osborn's students at Princeton and later at Columbia, Oliver Strong, and it was brought to successful conclusion in his dissertation published in 1895. (Oliver S. Strong. The cranial nerves of the *Amphibia*. *J. Morph. 10:* 101–230, 1895.) In this period, while still an undergraduate at the University of Cincinnati and later at Denison, I was fumbling with the same problem. Upon the appearance of Dr. Strong's thesis it was evident that his method was practicable and fruitful and I resolved to work with him. During the academic year of 1896–7 I sat vis-à-vis with Strong at a table in the Columbia zoological laboratory." (C. JUDSON HERRICK. The cranial nerves. A review of fifty years. *Denison Bulletin, Journal of the Scientific Laboratories. 38:* 41–51, 1943.)

As Professor Herrick's work progressed, he became increasingly devoted to the relations that must obtain between operations of the brain, the internal experience of the individual concerned, and the outward expression of these transactions in behavior, including social behavior. He was always alert to the psychological meaning potential in every observed anatomical relation. "What is the relation between bodily structure as mechanism in action and the mental life which unifies and controls this activity?" This is a question which calls for more facts; facts concerning bodily processes, subjective experiences and behavior. These facts appeared to Professor Herrick to lead directly toward the establishment of an ethic without dogma, an ethic freed of external authority, an ethic based entirely on scientific principles.

During 40 years, Professor Herrick enjoyed the close intellectual and scientific companionship of George Ellett Coghill who devoted himself to the behavior of developing primitive vertebrates, particularly *Amblystoma*. He also bore strong scientific ties with Professors Karl Lashley, Donald Hebb, and others interested in relating brain, mind and behavior.

Professor Herrick and his friend Coghill discovered in the neuropil one of the most important mechanisms for integration. Its first appearance in the *Amblystoma* embryo was associated with the development of the capacity to swim. They looked upon the neuropil as responsible for the marvelous and dynamic modification of intrinsic activities by use and by the environment. They conceived the neuropil to undergo continuing growth and adjustment for as long as learning is possible.

The first Symposium paper introducing original work was presented by Professor Clifford T. Morgan. Unfortunately, for reasons beyond his control Professor Morgan was unable to provide a manuscript for publication at this time.

". . . it is emphasized that in these amphibian brains the histological texture is generalized and probably at a primitive level of differentiation. The cell bodies of most of the neurons are densely crowded in a central gray layer next to the ventricle, from which their dendritic and axonic processes extend outward into the overlying white substance, where they branch and interweave to form a dense fibrous fabric, the *neuropil*. . . .

"*All neuropil is a synaptic matrix*. Here every contact between an unmyelinated axon and a dendrite or a cell body is a synaptic junction. In general, every axon contacts many dendrites and every dendrite and cell body has contact with many axons which may come from diverse sources. . . .

"This neuropil of primitive brains is regarded as the primary apparatus of integration. . . . All parts of the brain are . . . bound together by a web of conductors which is relatively undifferentiated, though by no means equipotential, for each area of neuropil has its own characteristic peripheral and central interconnections. . . .

"In the search for the organic basis of the integrative functions, of discriminative responses, of 'choice' (conscious or unconscious) of appropriate responses to particular excitations, and of that modifiability upon which depend memory and learning and all semantic activities (again conscious or unconscious), the presently available methods of neuroanatomy are obviously inadequate to do more than map the coarser features of the field to be explored. Yet this is an essential prerequisite for intelligent application of chemical, physiological and psychological techniques with minimum waste motion and futile groping. Here the study of developmental history—ontogenetic and phylogenetic—yields crucial data accessible in no other way." (C. JUDSON HERRICK. Apparatus of optic and visceral correlation in the brain of *Amblystoma*. *J. Comp. Neurol. 37:* 97, 98, 105, 1944.)

Neurophysiological studies on learning and motivation

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I HAVE BEEN TOLD THIS IS THE FIRST TIME IN HISTORY that the American Physiological Society has sponsored a Symposium in which motivation and learning, two of the so-called higher functions of the nervous system, are to receive specific, explicit and exclusive consideration. Whether or not this information is correct, there cannot have been very many previous symposia like this one. Up until a very few years ago most physiological measurements taken from brain or body seemed at best very remotely related to learning, motivation or any other higher functions of the brain, and our Society is not known to encourage mere speculation and supposition in public places.

Within the past few years, however, some astonishing developments in methodology, industriously applied to normal animals and people, have at last yielded measurements on brain function that seem to fill the void. Physiologists and psychologists in reasonably large numbers have begun to penetrate that bony shell within which nature hides the brain so securely, and their efforts are resulting in precise data on the neural events that occur when animals behave. My purpose here is to review some of these studies, and to relate their data—insofar as it is possible to do so at this early date—to learning and motivation.

ELECTROPHYSIOLOGICAL STUDIES

The electrical activity of the brain can readily be recorded, as everyone knows, through electrodes merely pasted to the scalp. However, when the electrodes are placed directly upon the brain surface, or even inserted into its depths, the brain waves become larger in amplitude (even though derived from a smaller number of cells), and more stable in form since unwanted artifacts are eliminated. Electrode proximity to the source of potential makes this generalization true, but such technicalities need not divert us here. The essential point is this: wires implanted upon or in the brain are for several reasons far superior to scalp electrodes, and when such electrodes are permanently glued to the skull and attached to a suitable plug through which connection to an EEG machine can be made any time the experimenter desires, the most reliable preparation for brain-wave recording will be achieved.

Solving the many problems involved in successful electrode implantation is commonplace knowledge in dozens of laboratories throughout the world today, whereas ten years ago very few knew how to do it at all. This simple fact holds the key to much of the research activity leading to this symposium.

Implanted electrodes permit examination and repeated examination of electrical events originating in a given brain location during a significant portion of the life time of an animal. But besides this, they will equally readily conduct electricity from outside in, and so the experimenter may apply electric shocks to any local brain region whenever he wishes to do so. Thus it is largely because chronic implantation of electrodes permits reliable brain-wave recording and brain stimulation studies that publications about brain function in behavior have begun to appear in such large numbers.

BRAIN STIMULATION

This chronic implantation technique is not really new, and its roots lie in the distant past. Long before the modern era of plastic plugs, stainless steel screws and dental cement, Baer (1) illustrated a device to be screwed into a dog's skull through which the cortical surface might be electrically excited. Actually, according to the Soviet physiologist Koshtoiantz (2), the first scientist ever to implant chronic electrodes successfully was a Russian named Simonov in the year 1866.

Most historians however cite 1870, the year Fritsch and Hitzig produced contralateral limb movements by applying shocks to the frontal cortex, as the year in which the progenitor, intellectually and technically, of our modern brain stimulation efforts appeared (3). Throughout the 90 years intervening since that experiment, the main question it raised has been a matter of controversy. Does the brain truly possess "centers" in which special functions are somehow localized? Today the answer "yes" seems to appeal to increasingly larger numbers of biologists, and much of the most useful experimental evidence for the affirmative stand comes from studies using implanted electrodes for brain stimulation. Let us examine a typical one of them.

THE THIRST CENTER

It is an elementary observation that animals throughout life drink exactly the amount of water required to

compensate for what they lose. This precise regulation is not accidental, of course, and therefore they must somehow be neatly constructed to that end. It turns out that the brain mechanism responsible for this remarkable behavioral response, drinking what is physiologically required and no more, can readily be disrupted by electrical stimulation of the hypothalamus. Andersson and McCann (4) demonstrated this to be true in several studies with goats. Each of two such animals, in good water balance, was made to drink an excess of water equal to some 40 per cent of its body weight within a few hours merely by delivering electrical shocks to its brain through implanted electrodes. The shocks that compelled them to gorge themselves in a manner entirely foreign to normal goats were delivered to a particular and restricted portion of the hypothalamus, the same one, incidentally, from which excessive drinking could also be induced by microinjections of hypertonic saline. Stimulation of no other region, including several very close by, produced the polydipsia. These experiments thus very reasonably define a true "center," a restricted brain location which, when activated, provokes a specific behavioral response.

The drinking response induced in the goats was not "simple," as were the muscle movements discovered by Fritsch and Hitzig. The stimulated goats searched for, moved toward, found, lapped, and finally swallowed the water they did not need. When taught prior to electrode implantation that they must first climb steps in a certain manner to get the water—a conditioned response—they flawlessly executed this complex reaction also when stimulated (5). Unquestionably some tiny hypothalamic region aroused by the shocks possesses easy and unique access to those brain mechanisms that produce their drinking behavior.

It is simplicity itself to move, if one is so inclined, from the experimental facts to the idea that the small hypothalamic region normally "monitors" body water level, "decides" whether drinking behavior is called for and can "order" the entire animal to take appropriate action. In short, one may conclude that a center for thirst exists. Its exact size, connections, and mode of action remain to be settled (6), but its participation as an important factor in motivating behavior seems established beyond question.

SELF-STIMULATION

If from the foregoing experiment we learn that a person need merely push a button to produce complex goal-directed behavior, from the following one we discover that an animal will come to push the button himself when by so doing he obtains shocks to his brain. This last fact, one of the outstanding neurophysiological discoveries of the past 10 years, has implications the full elaboration of which will carry us several long strides toward understanding motivation, learning, and still other features of complex behavior.

As with so many great discoveries, this one also came as a surprise, and by accident. Olds and Milner (7), studying learning in rats, placed within reach of an animal bearing implanted electrodes a lever which, when pressed, would close a switch and cause shocks to be delivered through the electrodes into its brain. Common sense predicts such a rat will either ignore the lever completely or, having pressed it once by chance, never go near it again. What started out to be a minor but necessary little control study ended up, however, as the first experiment of an important new research program, for once the rat did find the lever he refused to stop pressing it. Guinea pigs, cats, and monkeys have since been shown to behave in the same compulsive way. They will busily search out a hidden lever and accept punishment they do not have to take as they move toward it. On finding it at last they will if permitted manipulate it for days at a time, foregoing food, drink and even sleep as they work diligently to cause shocks to be delivered into their brains. What motivates these animals to do such unheard-of things? Is it some exquisite pleasure they receive, as several students of the problem staunchly contend, or the feeling of utter and complete well-being as others claim?

These suggested explanations, and most of the others advanced, appeal, one notes, to a class of body phenomena physiologists traditionally have rarely undertaken to study, at least as far as one can judge from a look at the table of contents of the textbooks they use in their teaching. When has motivation and the emotions, feelings, and similar vague body states ever before seriously entered the realm of scientific discourse in physiology? To be sure, physiologists have puzzled over, written about, and a few have even experimented in this area of what most of us call "psychology," but not with the widespread effort upon creative exploration of the brain mechanisms involved that has characterized the past 10 years. Undoubtedly we shall be hearing even more of this kind of work in the near future.

LEARNING AND THE EEG

Some 18 months ago the Soviet Academy of Sciences sponsored in Moscow an International Colloquium on the EEG and Higher Nervous Activity. The published proceedings of the Conference (8) provide for anyone interested in the brain mechanism of learning as good a summary of world-wide views on the matter as exists anywhere. This Moscow Colloquium is a sample of many recent attempts to discover how far the study of brain waves has taken us toward understanding the neural events that transpire when animals learn. One could not hope to summarize the field they cover here, and so I shall merely describe a single experiment drawn from our own laboratory experience to illustrate how such studies go.

The experimental subjects are cats and monkeys, all with wire electrodes implanted permanently in cortical and subcortical brain tissue (9–11). From time to time they are brought to an experimental room where these

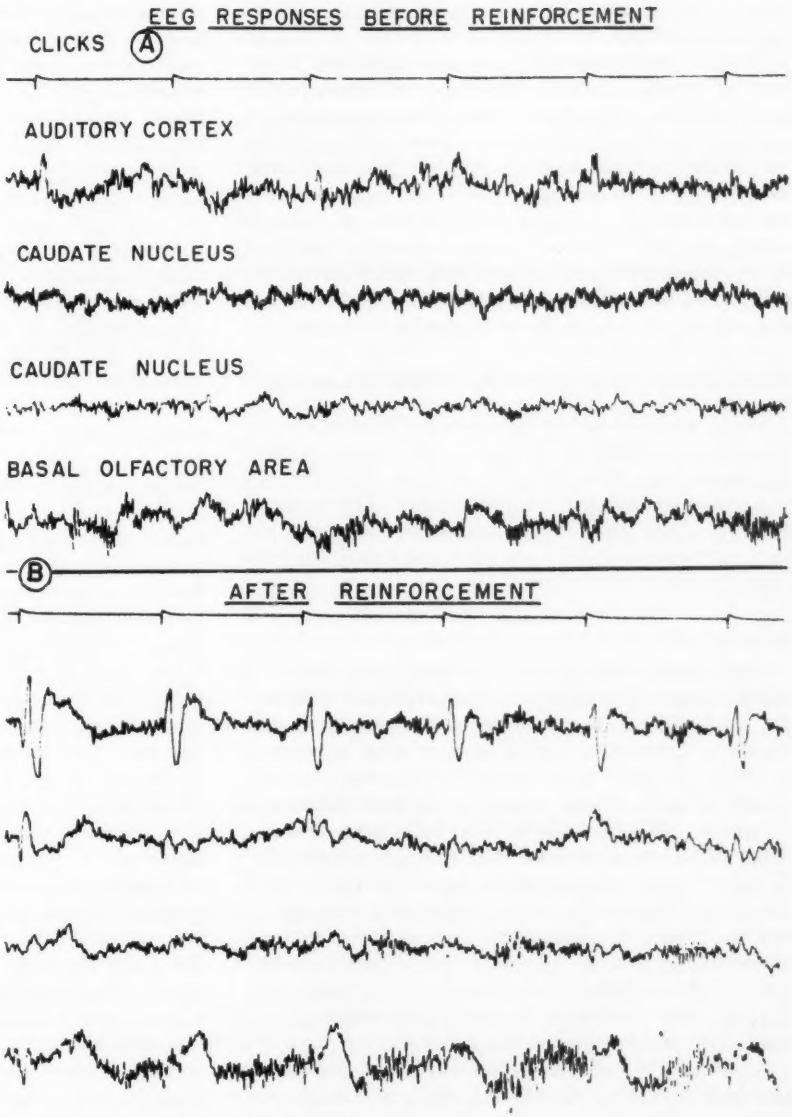

FIG. 1. Response to clicks before and after reinforcement, electrodes implanted in cat brain in areas named in upper record, *A*. Clicks coming 1/sec. evoke little brain response when meaningless (*A*), but large response when meaningful a few minutes later (*B*).

wires are attached through a plug to suitable EEG recording devices. Our interest is not in the spontaneous brain rhythms thus made recordable but in the pattern of electrical waves evoked from the brain by a sensory stimulus such as a click or flash of light.

This evoked-response pattern fluctuates in amplitude and varies in duration as the animal spontaneously passes through, or is experimentally taken through, various behavioral stages. For instance, if the resting animal is exposed overnight to a click monotonously repeated every few seconds, its brain produces a modest-sized EEG reaction to that click the next morning (fig. 1*A*). A familiar signal—the click that neither threatens ill nor promises good—provokes little reaction from the brain. But now let us arrange a rubber tube so that from a distance we may if we choose blow a puff of air into the animal's face whenever the click sounds; the essentials for a simple Pavlovian conditioning experiment will thereby be realized. The click (conditioned stimulus) when reinforced by the mildly noxious air-puff (unconditioned stimulus) becomes meaningful as a signal for the advent of an unpleasant event, or so it appears as we watch the animal's behavior and introspect about our own reaction to a similar situation. The simple experiment regularly works, for when a puff of air is paired with the click several times, the click turns out to produce an EEG reaction that is large in amplitude and prolonged in duration, indicating considerable enhancement of the brain activity it sets into action (fig. 1*B*). This state of enhanced brain activity is not permanent, however, for if we stop puffing but continue to deliver the click every few seconds for hours on end, the brain

responses to them decline with time to the modest control size once more.

This rise and fall in EEG response-amplitude correlated with conditioning followed by extinction in the simple Pavlovian paradigm occurs throughout the brain, not merely in the cerebral cortex where such events were once thought exclusively to take place. The thought that for learning to occur widespread brain areas probably become involved in a particular sequence and order is merely one of the many new insights emerging from such EEG work carried on in experimental laboratories over the past few years. Dr. Adey's presentation later in this Symposium will elegantly illustrate some others.

PHYSIOLOGICAL SUBSTRATE FOR MOTIVATION AND LEARNING

Severe problems face anyone who wishes to synthesize a satisfactory model out of modern information such as that on centers, self-stimulation, and the EEG changes in learning we have just briefly examined. The problems to be faced are by no means new ones, and they have been stated in many different ways, but I shall rephrase them once more nevertheless.

NEUROPIL ORGANIZATION

What are the mechanics by which a given collection of cells acquires and displays the properties of a "center?" Just exactly how does a group of hypothalamic cells become organized during embryology so as to regulate drinking behavior while another apparently similar group in the medulla comes to regulate breathing? C. Judson Herrick, whom this Symposium honors, spoke repeatedly of the "unknown mechanical principle" at work within such restricted regions of the nervous system, and his book (12) and the paper accompanying this one eloquently present his views. We need most urgently to know what goes on in those chaotic bramble-patches of dendrites, axon terminals, cell bodies and glia revealed by every electron microscope picture ever made of the central nervous system.

Apparently no one has as yet discovered the special biochemical and/or biophysical keys to unlock the mechanics of this neuropil organization. When he does he will have some splendid opportunities to test Herrick's ideas that the neuropil is involved both in the innate responses of organisms (e.g. drinking, in goats) and in the ones they acquire through learning. As an example of what pleasures lie in store for him consider, for instance, the recent report of Myers (13) that a chimpanzee with corpus callosum and anterior commissure cut can readily learn to solve a complicated puzzle—but only with the one hand he was permitted to use in the training period. Given a chance to solve the same puzzle with the untrained hand (which a normal animal can do at once) he fails completely. Evidently the cortical hemisphere contralateral to that of the trained hand has been brought through this simple experiment into a state of organization which is entirely absent in the cortex on the opposite side. Some specific change created in one half of the brain through learning simply does not exist in the other half. If Herrick is correct, there is a significant difference in neuropil organization in the left and right cortical hemispheres of Myers' chimpanzees. Who, we may ask, will be the first to define and measure this difference?

INFORMATION TRANSMISSION

Besides discovering what the physical and biochemical basis of "centers" may be, neurophysiology faces the question of how the different brain regions so defined are interconnected and mutually influence one another. The goat set to drinking by electrical shocks applied to its hypothalamus does so because one neuropil region has direct and easy access to a distant one where the organization of motor responses appropriate for drinking behavior is somehow represented. What is the nature of this connection between one brain region and the next?

Traditionally, neurophysiologists answer this question in two ways. They recognize, first of all, humoral factors both at the synaptic level where transmission is effected from pre- to post-synaptic neurons, and at what might be called the neuropil level where dissolved substances brought to the region via the blood determine whether or not the "center" will display its characteristic activity. The goat can be fooled into drinking more than he needs, one recalls, also by hypertonic saline injections into the hypothalamic thirst center, a fact that virtually proves the peculiar and specific chemical sensitivity of this particular cellular aggregate. Of what this sensitivity consists, and how a reaction in such cellular aggregates to fluctuating blood levels of this and that substance becomes converted into organized motor responses are old problems, but they remain vital issues for neurophysiology. The many special sessions on Neuropharmacology on the Federation program of which this Symposium is a part testify to the lively current interest in these matters.

Besides these chemical routes by which information is transmitted to and within the nervous system, physiologists point of course to the neurons that stretch from one nuclear mass to the next and conduct physiologically all-or-nothing impulses in one of the two possible directions between them. Neuroanatomical data are crucially relevant here, and I should like now to show and discuss a much simplified diagram portraying our present knowledge of the routes interconnecting the various brain regions. Figure 2 shows, at the left, the sensory inflow from the body surface distributing itself through the brain chiefly via the classical afferent pathways, on the one hand, and the more medial brain-stem regions ("reticular formation") on the other. Both routes eventually terminate in the cerebral cortex, and much speculation attaches to what their separate contributions may be at that "highest" level where, as Dr. Sperry will develop shortly, we have excellent reason to believe neural events vital for at least some kinds of learning take place.

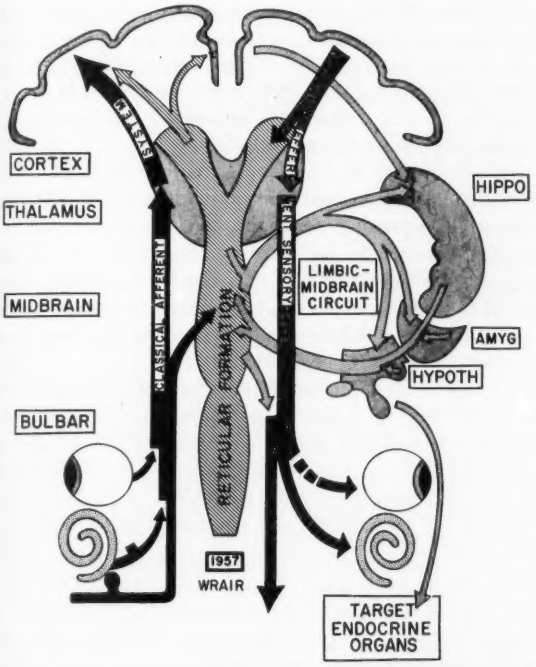

FIG. 2. A schematic anatomical diagram of the mammalian brain. Three major systems are shown: *1*) Fiber bundles passing from and to the sense organs (here labeled classical afferent, and efferent sensory); *2*) medial brain-stem structures (reticular formation); and *3*) the limbic-midbrain circuit through which paleocortical structures (hippocampus, amygdala), hypothalamus and mesencephalic structures intercommunicate. Text discusses relation of these systems to motivation and learning.

On the right of the diagram appear two additional neuronal systems—the efferent sensory and the limbic-midbrain circuit. The first of these, the so-called efferent sensory pathway, has been under intensive study for several years but its function is still largely a mystery. Since these efferent (or descending) fibers largely originate in and distribute to the nuclei of the classical pathways the idea that they somehow regulate the incoming traffic from eye, ear and other sense organs has received serious consideration (14). The thought here is that the nervous system may be so constructed that it can decide for itself, so to speak, what aspects of the immediate environment it will permit to enter for processing. Goal-direction (another word for motivation in animal behavior) means selecting one course of action from among several possible ones, and this selection is performed in part by emphasizing certain sensory inputs at the expense of others. The possibility that the efferent sensory systems provide a means by which animals do indeed emphasize one sensory input over another in the common experience of attention ("looking," "listening") is an exciting new dimension we can confidently expect further investigation to explore.

The limbic-midbrain circuit shown in the diagram has emerged within the past ten years as still another anatomical and physiological entity having important implications for behavior. Anatomically the circuit consists, in essence, of a loop of fibers extending from medial midbrain regions into forebrain nuclei and then back again. Synaptic interruptions, particularly in the hypothalamus, characterize both the ascending and descending limbs of the loop, and thus ample opportunities exist within it for integration of neural activities arising in many nuclear masses. Input from cingulate neocortex, which in turn receives afferents from the anterior thalamic nucleus, draws still other brain regions into the large loop, much of which has been known to anatomists for many years (15).

Several developments have provided the impetus for current interest in this limbic-midbrain circuit, and these are adequately reviewed in the Handbook of Physiology (16). Physiological ablation, stimulation, and EEG studies implicate its nuclei and fiber tracts in a wide range on body processes. The thirst center, for instance, is embedded within the stream of limbic-midbrain fibers as these flow through the hypothalamus, and the self-stimulating animal works hardest and most reliably for shocks through electrodes implanted in some component or other of the limbic-midbrain circuit. The contributions of hippocampus during learning have for several years been under intensive EEG analysis as has the blood level of hormones under control of the neurohypophysis, itself a component of the large loop. The limbic-midbrain circuit, in short, is at once an anatomical entity, a conceptual framework, and a rich source of new data about brain function. To it comes information from all the sense organs (and in regions like the mesencephalic reticular formation and amygdala, light, touch, and sound stimuli all may drive a particular single cell), while out of it go the messages required to control numerous aspects of behavior (as illustrated by the goat forced to drink). It can be viewed as a brain-within-the-brain, the repository of those mechanisms that enable a species to display its characteristic behavioral traits and the means by which cerebral cortex is prepared for whatever specific changes it undergoes during learning. Perhaps this view overstates the case, but for many students of motivation and learning it will doubtless continue for some time to supply new and experimentally testable hypotheses.

RECAPITULATION AND SUMMARY

The decerebrate or anesthetized animal cannot learn nor is it motivated, and hence the conventional physiologist of 10 years ago was barred from studying these matters even if he would. An unconventional physiologist, like C. Judson Herrick, who would study them, lacked the technical wherewithal to do so. Within the past 10 years however several technical advances, including safe and certain ways to implant electrodes and other devices, allow the brain of fully aware animals to be brought under detailed scientific scrutiny. Brain wave, ablation and electrical stimulation experiments conducted on such preparations combine with a flood of

new anatomical and biochemical data about the brain, to yield a veritable Niagara Falls of new information relevant to our problems. The task of relating these new facts to well-established old ones is a major intellectual effort. What old conceptions must we abandon and what are the outlines of a satisfactory conceptual framework that will organize these modern data from psychology, physiology, and anatomy?

The ancient idea of centers—local brain areas in which specific and particular functions have special representation—appears to gain rather than lose ground. The brain cannot be merely a collection of equipotential cells, and we have examined several dimensions along which one collection of cells turns out to differ from another. An assembly of hypothalamic cells—the thirst "center" for instance—may display specific chemical sensitivity, and when electrically activated produce complex organized behavior—in this case acquisition of water for drinking. We have not discussed temperature, hunger, respiratory or sexual centers, but they appear in principle to behave similarly. Another group of cells—localized, say, to part or all of one cerebral hemisphere—can be made to acquire a very specific organization necessary for performance of a complex manual task if an animal is given training and experience. Still another—the medial forebrain bundle of the limbic system, for example—produces a unique commotion within the brain of the self-stimulating animal, or at least so we must infer from his preoccupation with the lever-pressing that delivers him the shocks he works so diligently to obtain. These physiological and behavioral studies seem to be bringing specific brain sites important for motivation and learning more sharply into focus.

Other experiments lead us toward the conclusion that several such regions participate, and in some orderly, temporally-dependent sequence, to produce the specific changes responsible for learning. Somehow the brain prepares itself for a change it is about to undergo, and the EEG studies currently being prosecuted in such large numbers yield much new information for sophisticated speculations on this preparation process.

What we call motivation and learning will obviously be understood as soon as we understand the fundamental mechanisms by which information is stored within and retrieved from the aggregates of cells making up a central nervous system. Herrick's view, that detailed study of the biochemistry and biophysics of the neuropil, offers the greatest promise of bringing us to this goal, never made more sense than it does today. One can only regret that the vast armamentarium of modern physiology, psychology, and anatomy was not available to Herrick when, years ago, he was prepared to subject his cherished notion to critical experimental tests.

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16. *Handbook of Physiology*. Baltimore: Williams & Wilkins, 1960, Vol. II, Chaps. 52, 55–58, 61, 63.

"Attention was first directed to the peripheral nerves and their end-organs, which are, of course, most accessible for experiment, and we now have much exact information about what is going on in nerve fibers of various sizes and structure during nervous conduction. Most of those peripheral nerve fibers connect with the central nervous system and the part that lies within the spinal cord or brain probably performs in much the same way as the peripheral part. But those physiologists who hope to explain the functions of the central adjustors in terms of the laws of conduction observed in peripheral fibers and nothing more are deluded. The central structure is radically different and no mechanical model has yet been devised which seems adequate to perform the functions observed in the behavior of men, monkeys or rats. All of these animals condition their reflexes, learn by experience, exhibit behavior which objectively we would call rational, can abstract from mixed experience its common features, and show types of spontaneity and initiative which conform with none of our standard categories of behavior. A rat, so far as we know, has no abstract ideas, say of a triangle, but he can and does distinguish a triangle from other geometric figures and recognize it, regardless of size, position or illumination. Operationally considered, this is an abstraction." (C. Judson Herrick. The cranial nerves: a review of fifty years. *Denison University Bulletin, Journal of the Scientific Laboratories 38:* 42, 1943.)

"The reason for the decussation of the major conduction pathways of the somatic sensori-motor systems has puzzled neurologists for many years, and fantastic theories have been expressed, some bearing the names of great masters—Wundt, Flechsig, Cajal, and others. . . . None of these speculations have yielded satisfying conclusions." (C. Judson Herrick. *The brain of the tiger salamander, Ambystoma tigrinum*. Chicago, Univ. of Chicago Press, 1948, p. 289.)

Some developments in brain lesion studies of learning[1]

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THE FOLLOWING IS A RAPID SURVEY of some of the things we've been doing in recent years in following up what for present purposes may be called "the split-brain approach to the learning problem." The general idea involved is simple enough: we first split the brain longitudinally down the middle to separate the right and left hemispheres, one of which is then reserved for the use of the animal, while the other is donated to science for application of the ablation and other methods of analysis.

In the surgery, we first sever the large corpus callosum, the main connecting link between the hemispheres. Along with this we generally cut also the smaller hippocampal and anterior commissures (see fig. 1). If vision is to be involved, the optic chiasm is divided in the midline or one of the optic tracts. For other purposes the following may also be sectioned: the massa intermedia, the habenular commissure, the posterior commissure, the quadrigeminal plate containing the commissures of the colliculi, and, in some of our more recent cases, also the cerebellum. The fibers of the small supraoptic commissures are presumed to be cut also.

This is as far as we have carried the midline sections to date, but this does not necessarily represent a limit from the surgical standpoint. It would be quite feasible to continue cutting into the upper end of the tegmentum, but here one soon begins to encounter important decussating fiber systems that connect structures up and down the brain, the cutting of which, of course, is quite a different thing from the cutting of commissural cross connections between homologous pairs of control centers.

It can be seen that these midline sections, except for loss of part of the visual inflow, leave nearly all the sensory, motor and other brain-stem relations intact. Also the great bulk of the internal organization of each hemisphere is preserved. Accordingly, the two separated hemispheres following recovery from the surgery continue to carry on most of their regular functions and the subsequent behavior is remarkably normal in character. In fact, monkeys so operated upon are hardly distinguishable from their normal cage mates under most ordinary circumstances, especially if the cerebellar section is not included. One does not notice any paralyses, spasticity, ataxia, no forced circling or other asymmetries, no hyperactive pacing nor apathy, automatisms nor mutism, nor have we observed to date any autonomic dysfunctions. Some slight changes in eye movements may be noticed in cases with section of the posterior commissure and/or quadrigeminal plate. Those with the cerebellum divided have shown some weakness and unsteadiness especially noticeable during the first months after surgery but it diminishes gradually. For the most part the monkeys appear normal and are quite suitable for behavioral testing.

However, when one studies more carefully the learning and memory of such "split-brain" animals under special training conditions where the inflow of sensory information to each hemisphere can be separately restricted and controlled, it is possible to show that each of the divided hemispheres now has its own separate gnostic or cognitive system: its own separate perceptual, learning, and memory processes. It is as if neither hemisphere any longer has any direct knowledge of what the other is doing, nor any direct memories of what has been going on in the other hemisphere subsequent to the time of section.

Although there were some intimations that this might be the case in the earlier literature on the corpus callosum, they were outweighed by confusing and contradictory observations (1). The first convincing demonstration was obtained by Ronald Myers in a nice series of experiments beginning about 1953 (16) which he carried out on the functions of the corpus callosum and on interocular transfer in the cat. In brief, Myers showed that a cat with midline section of the optic chiasm and forebrain commissures is unable to remember with one eye visual pattern discriminations learned with the other eye (12–14). In fact, with the second eye such a cat easily learns a discrimination habit that conflicts directly with what it had just previously learned with the first eye.

At this point the question immediately arose as to how separate in their function the two hemispheres really are: For example, might it be that the unblinded side in a commissurotomized cat is simply much more dominant than is usual with the corpus callosum intact and tends to drain the attention off to the unblinded side? In partial answer to this we find that extensive cortical removals

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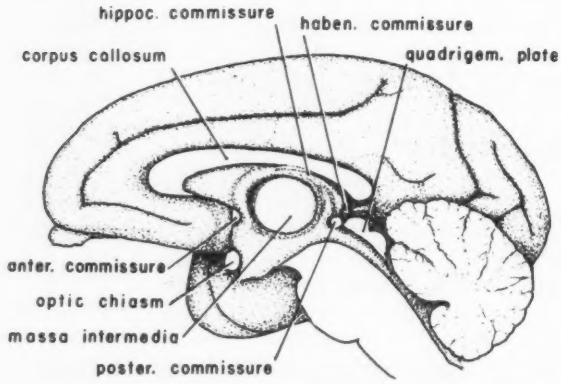


FIG. 1. Midline structures sectioned in surgical splitting of brain into right and left halves. Division of cerebellum has been added to above in some experiments.

on the unblinded side, that would be expected to depress severely any dominance of this hemisphere, still do not force visual learning of pattern discriminations over into the other separated hemisphere (20, 24). Also, when we compared the learning curves of the second eye with those of the first eye on a statistical basis, we found no significant carry-over from the training of the first eye to that of the second (25). Thus the visual learning of the second eye seemed to benefit in no way from that of the first. It was as if in using the second eye, the cat had a selective visual amnesia for the learning experience with the first eye.

The training and testing in these experiments is carried out in a type of discrimination box that I had designed earlier for studies of detailed pattern perception in the cat. The basic principle involves putting the animal inside a darkened box where the only light enters at one end through a pair of translucent stimulus patterns that the animal is being trained to discriminate. The cat walks up to and pushes its head into the stimulus figures, mounted in swinging doors, the correct one of which will open and give access to a bit of food on a tray outside the box. And the final technical point—the stimulus figures are of a size roughly the same or slightly smaller than that which will admit passage of the animal's head. Whatever the critical factors, the apparatus has enabled us to get extremely refined pattern and size discriminations, and to quantify the cat's perceptual performance with test scales based on graded reduction of dissimilarity. Vision of one eye is occluded during the training sessions by having the cat wear a rubber eye patch as devised by Myers.

Let me emphasize at this point that I am serving pretty much as a spokesman here, for work that has been very much a group effort. In addition to Myers who got the project off the ground to a good start, there have since been a dozen or so research fellows, graduate students and assistants who have all made their important contributions.

In another apparatus engineered by John Stamm, we found that the same kind of functional independence prevailed in the separated hemispheres with respect to the learning of somesthetic discriminations that involved touch and pressure on the surface of the forepaw (26). The cats were trained to push the correct one of two pedals which they were unable to see and had to discriminate entirely on the basis of touch. Again, a comparison of the learning curves revealed that the strong transfer of training from the first paw to the second in normal control cases was entirely lacking in those cats in which the corpus callosum had first been sectioned. This lack of transfer applied as well to the motor learning that was involved in gaining proficiency in the operation of the pedals. Hence it was inferred that in the split brain cat "the left forepaw knoweth not what the right forepaw doeth."

With respect again to learning and memory in the visual sphere, the findings in the cat have been confirmed also for the monkey by both Dr. Downer (2) and myself (21) quite independently. Perhaps it should be mentioned here that the attempt to test the effect upon visual learning and memory of combined section of both the optic chiasm and corpus callosum is something that has been tried a number of times in the past and abandoned, largely because of the surgical problems. The present-day success can be attributed in large measure, in our own case at least, to the carry-over to the mammalian brain of the surgical procedures of experimental embryology adapted originally to salamander larvae and frog tadpoles. In the main this involves the use of a high-powered stereoscopic microscope for most of the finer aspects of the surgery along with correspondingly delicate instruments. In our own case it also includes adaptation of the operating table for use in the sitting position, not only because of the fatigue factor, but also for the increased support, steadiness, and prolonged concentration that it favors.

The monkey is much less inclined to be cooperative than is the cat about the wearing of an eye patch, and accordingly I devised a training box by which we can restrict vision to one or the other eye. Essentially it has two eye apertures so positioned with reference to head restraints that one of the peepholes is accessible only to the right eye, and the other only to the left eye (fig. 2). Either or both eye apertures can be opened or closed from trial to trial. In addition, there is a sliding panel that controls the use of the arms and allows the pairing of either eye with either hand from trial to trial. This has the advantage over the use of an eye patch, corneal occluders, or sewing of the eyelids in that it is possible to switch easily back and forth from one eye to the other in a given series of trials.

One can give a few trials to the right eye and then a few trials to the left eye, and so on. If this is done while the monkey is in the process of learning two conflicting pattern discriminations with right and left eyes, it is possible to show that while one brain half is learning to select, say a triangle and avoid a square, the other hemisphere can be learning at the same time to do exactly

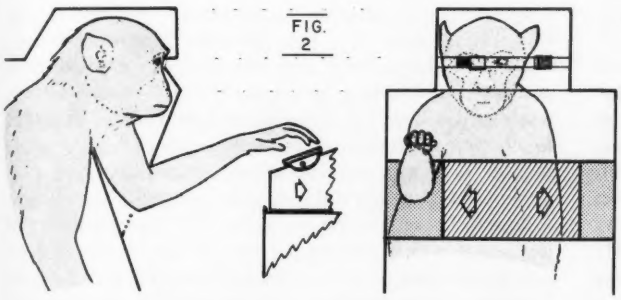
the reverse. Under these conditions the two learning curves then rise in parallel concurrently in the separated hemispheres, with no particular retardation nor cross interference apparent (23). Needless to say, the normal monkey brain does not operate in this way.

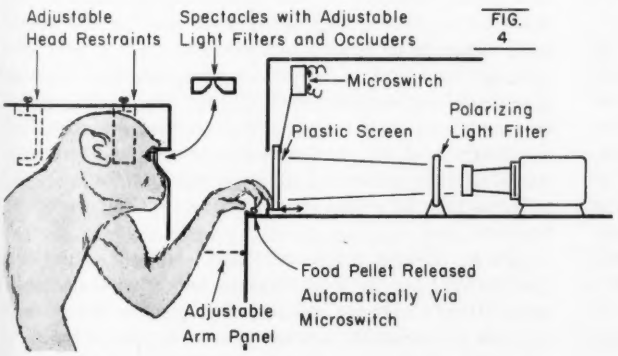
More recently we've been trying to carry this a step farther in an effort to find out if such reversed discrimination learning could proceed in the separated hemispheres, not only concurrently but really simultaneously. Instead of switching alternately from one to the other eye, both eyes are used on all trials and the training conditions are arranged so that each single trial feeds back conflicting data simultaneously into the separate hemispheres. To state the problem another way: does the split-brain monkey, while learning new visual habits under these conditions, have to pay attention to what's coming into only one eye at a time—or, can the monkey attend to what is coming into both eyes simultaneously, deal with both sets of conflicting information, file them in two separate memories, and so on thus learning simultaneously and in parallel the two reversed discriminations?

A method by which this can be tested based on the use of polarized light (27) had already been devised by Colwyn Trevarthen for studies on conflict. In his apparatus diagrammed in figures 3 and 4 the pair of patterns to be discriminated is shown through two projectors, one for each eye. Each of the projectors has a polarizing filter in front of it, the one filter set at right angles to the other. These two filters are interchanged at random, right to left, from trial to trial. Both sets of patterns from each projector are projected on top of each other on two plastic screens, one of which the monkey pushes in order to obtain a reward. Another filter in front of each eye cuts out the patterns from one or the other projector from trial to trial.

With this set-up the two plastic screens appear to one eye as if, for example, there is a circle on the left and a cross on the right, while to the other eye it appears just the reverse, as if the cross were on the left and the circle on the right. Thus, while one eye sees the hand reach out and push the circle on the left, the other eye views the same act as a reaching out and pushing of the cross on the left. Any kind of projectable figure can be used and the pairs of patterns can be simply different as well as reversed. Essentially the apparatus is a monkey automat; a correct choice activating a micro-switch that releases a peanut, dehydrated banana tablet, or other monkey delicacy.

The procedure is to allow learning to progress under these conditions with both eyes open until the learning curve reaches a level of 85 or 90% correct. At this point each eye is tested individually to find out if the learning has been taking place in one hemisphere only, or in both, and to what degree. In his tests to date involving three split-brain animals, Trevarthen has found that the proportion of learning found in the right and left eye varies: *a*) One eye may learn fully, the other only partly or not





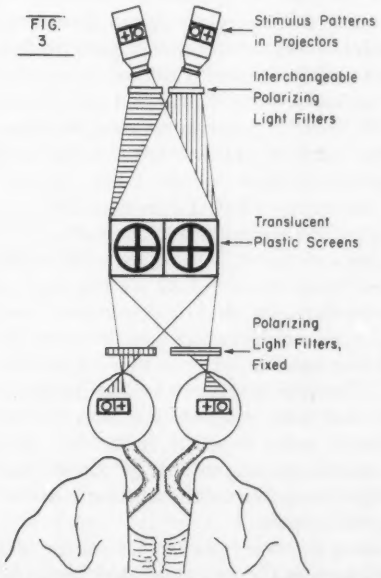


FIG. 2. Profile and front diagrams of visual discrimination training apparatus for controlling use of different eye-hand combinations.

FIG. 3. Diagram illustrating use of polarizing light filters to present different visual problems to right and left eyes simultaneously. Inversions produced by projector and ocular lenses are omitted

FIG. 4. Profile sketch of same apparatus illustrated in fig. 3.

at all. *b*) In some cases neither eye can perform the discrimination alone, but together they are successful, suggesting that different components of the same habit complex may be learned on right and left sides and that the neural changes of an engram may be complex and divisible. *c*) Each eye may fully learn its own problem. Although chance is largely against such an equal balance of the learning on both sides, occasional instances have emerged in which both eyes have fully learned, simultaneously, their opposed discriminations.

In other words, in these instances, in approximately the same order of time that it takes a plain ordinary-brained monkey to learn one discrimination habit, the altered, double-brained monkey may successfully master two such problems. This result is less favored during the first few problems than it is later after the monkey has become proficient in the use of the box and in the pairing of either hand with either eye.

This evidence of simultaneous reversed learning in the separated hemispheres, gives us some things to think about with respect to learning theory, and the role in learning of the attention process referred to above and of other components of learning, such as the motivational background and mental, perceptual and motor sets. Are all these constituents of learning duplicate, or perhaps bifurcate in structure, with prongs qualitatively different in the two hemispheres? The next step calls for tachistoscopic (quick flash) presentation of the stimulus figures to further reduce the attention span.

When the monkey has been trained through the left eye to select a circle and avoid a cross and through the right eye to do just the reverse, the two hemispheres get along harmoniously enough so long as what looks like a circle to the right eye appears like a cross to the left. But now suppose that we rotate one of the eye-filters 90°, so that each eye sees the same set of patterns from the same projector. With one brain trained to reach for the left plaque containing the circle and the other trained to reach for the right plaque containing the cross, what kind of mental conflict will arise? Will each hemisphere try to decide for itself, or will one follow the decision of the other? Actually, what Trevarthen finds is that the monkey in this situation may exhibit some extra hesitancy and indecision, but no extreme conflict. One or the other brain tends to take over to select consistently either crosses or circles. The dominance shifts from time to time from one hemisphere to the other as a result of factors that only in part can now be predicted or controlled. This would seem to be just another example of the fact that brain organization from the highest to the lowest level tends to be an "either/or" kind of thing. Either one excitation pattern prevails or another; but it is the rare exception that competing patterns produce a confused mixture.

So much for the evidence for independent learning and memory in the two separated hemispheres. Given now these two independent learning and memory systems operating in parallel, let's turn back to our original proposition: that is, to leave one of the learning systems for the use of the animal, while we take the other for experimental analysis.

Briefly, what are some of the kinds of things that have been and can be done with this approach? Especially what are some of the advantages of using the half brain for analysis instead of the whole brain? The advantages to the animal over the usual bilateral invasion are obvious; the gains are also considerable from the experimental standpoint. But first in this connection, it is worth our remembering that the half brain is, after all, nearly a whole brain in the sense that it contains a full complement of all the cerebral integration centers and cortical areas and all their interrelationships excepting only the right-left cross connections. Practically the entire pattern and most of the problems of cerebral organization above the midbrain level are there for the unravelling within the half brain.

One obvious advantage of the split-brain preparation lies in the factor of built-in controls—of many kinds, of the homozygous, identical-twin quality with additional common denominators in experiential factors—not only for the testing of learning and memory, but also for acute physiological studies as well. By restricting the sensory inflow to one side, or by other procedures that cause the animal to attend in, and use, one hemisphere; the effect of unilateral lesions, drugs, shock and anesthetic administrations can be checked and compared with the function of the normal side.

Another advantage lies in the possibility of investigating the role in learning of cerebral structures such as the caudate, the primary motor cortex, and others, the bilateral ablation of which produces incapacitating or other secondary effects that act to obscure or confuse possible contributions to learning. Each control center tends to be involved in a whole spectrum of different functions, and in many cases only the more basic impairments can be inferred after bilateral removals, the others being hidden or untestable in the presence of the former.

The split preparation similarly offers the possibility of using much larger ablations even to the extreme of removing most of the neocortex and working with isolated functional remnants. We have isolated the visual cortex of the cat in this way (see fig. 5) and found that extremely little vision survives such isolation (20, 24). A next step is to go back and to restore in other animals different portions of the removed cortex to determine the respective contributions of each to visual learning and memory. Comparison of the effects of temporal and frontal removals indicates that in the cat the temporal lobes are not as important for visual discrimination as they are in the monkey (11).

A very different result was found to follow similar surgical isolation of the frontal cortex including the somatic areas. In this case the isolated remnant was found to be capable of mediating excellent learning and memory of new somesthetic discrimination habits performed in a pedal-pressing apparatus (22). The elusive engrams or memory trace for these new habits would seem to have been, if not trapped, at least cornered within the local

FIG. 5

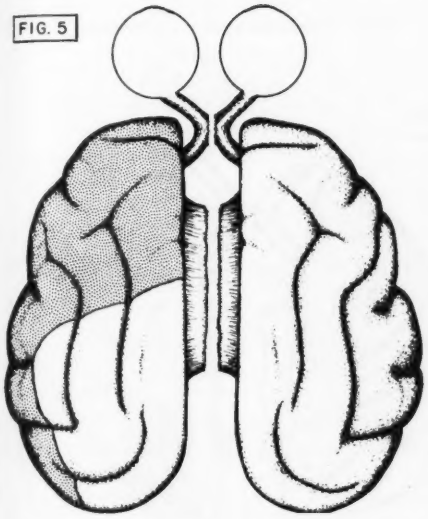


FIG. 5. Central visual cortex is surgically isolated in left hemisphere; right hemisphere serves as control.

FIG. 6

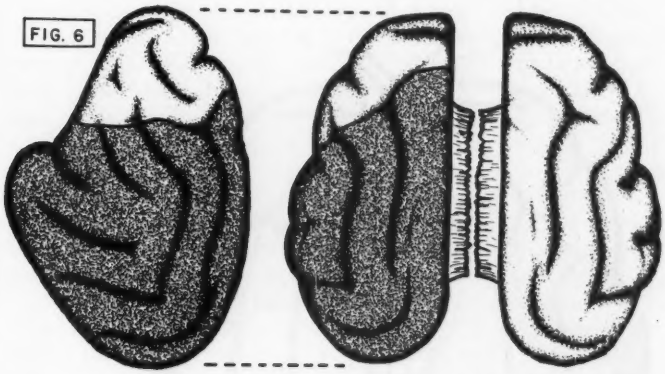


FIG. 6. Capacity for high-level somesthetic discrimination learning and memory survives isolation of the frontal cortex as shown

cortical area indicated in figure 6. It should be possible to further localize the engram by paring away the anterior, medial, and ventral edges of the area, and also to test the contribution of various subcortical centers that remain undegenerated like the caudate, amygdala, and hippocampus by prior placement of deep stereotaxie lesions. The preparation thus furnishes a promising means for determining the critical minimum cerebral apparatus essential for learning and memory of somesthetic discrimination in the mammalian brain.

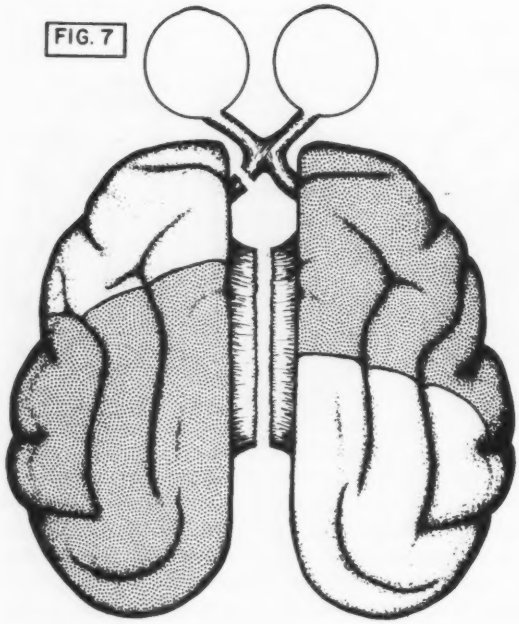
To further assure in the foregoing experiment that the habits were not being mediated by the contralateral somatic cortex, a complementary removal of the same area was made on the opposite side. To start making lesions in the hemisphere that was set aside for the use of the animal may not seem exactly fair, but at least a "trade" was made. At any rate, the feasibility of adding complementary lesion patterns in the intact hemisphere of the split brain offers further possibilities for the analysis of functional relationships.

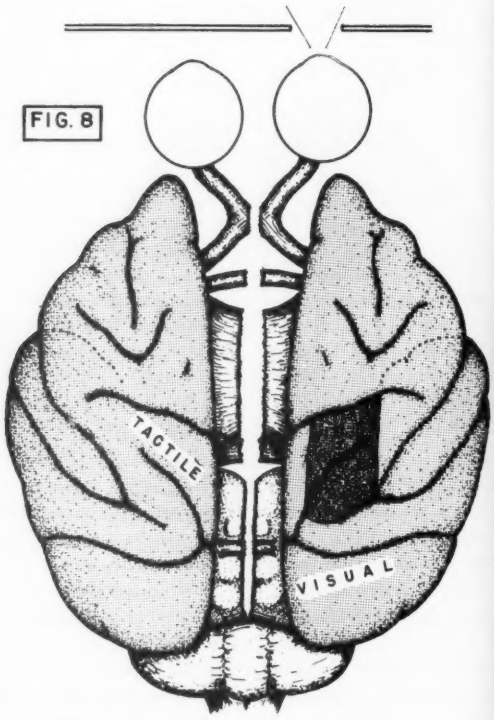
There are other promising angles in the foregoing "somatic island preparation" for the investigation of learning: For example, we could test its proven pedal-pressing learning capacity using visual or auditory instead of tactile stimuli; that is, we could ask, "Could such a cat learn to press a pedal that activates the correct one of two different tone patterns, first, with the auditory cortex in the opposite hemisphere?" If not, could it then do so if an isolated patch of auditory cortex were to be left on the same side as the somatic island? Should the answer still be no, then what kind of intracortical bridges and connections are needed to satisfy the learning requirements?

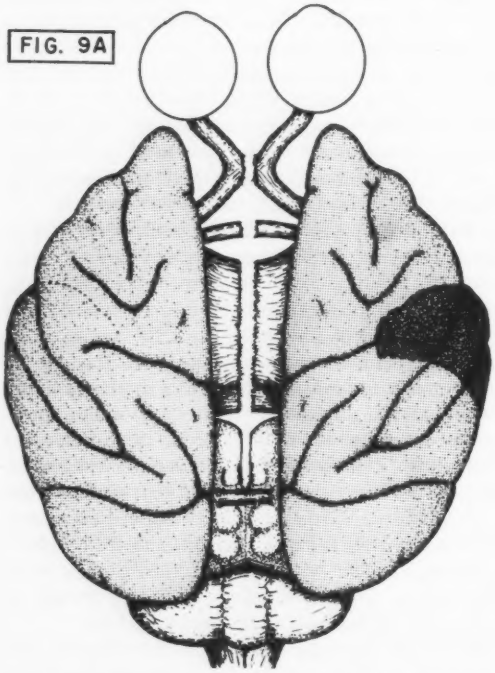
Figure 7 illustrates a basic type of complementary lesion preparation we've been using with a number of variations to study visuo-motor coordination. The experimental question here was: "Can visual information that is processed in one hemisphere serve as a guide for limb responses, the cortical centers for which lie in the opposite hemisphere and surgically separated from the visual inflow?" And, in another experiment with the same type of preparation, "Could a visual signal be used as the conditioning stimulus for a conditioned flexion response of the forelimb in question?" The evidence so far indicates that the answer will be "yes" to both of these questions (unpublished observations of R. E. Myers, R. W. Sperry and Nancy Miner, and of T. J. Voneida and R. W. Sperry), but there are qualifications and of course each new answer raises four new questions.

The visual guidance of a limb, the main cortical control centers for which lie in the hemisphere opposite that of the visual inflow presents certain problems. The split-brain monkey with unilateral vision shows a tendency to prefer to use the arm governed from the same hemisphere, i.e. the contralateral arm. However, this is only a tendency and it is easily overcome in a matter of hours in most of our training situations (23, 27, and unpublished observations of R E. Myers, R. W. Sperry and Nancy Miner). On the other hand, this tendency can be enhanced in other testing circumstances to the point where use of the ipsilateral arm may appear extremely difficult or impossible for a prolonged period (3). However, preference for the ipsilateral arm may prevail when the given arm is strongly dominant either naturally or as a result of training in a particular apparatus.

It was found that the split-brain cat readily uses either forepaw to carry out visual directives in the learning of visual discriminations with one eye (19). Monkeys also are able to use either hand under similar conditions, but there is a difference between the ipsilateral and contralateral performance, the contralateral arm having the advantage (27).







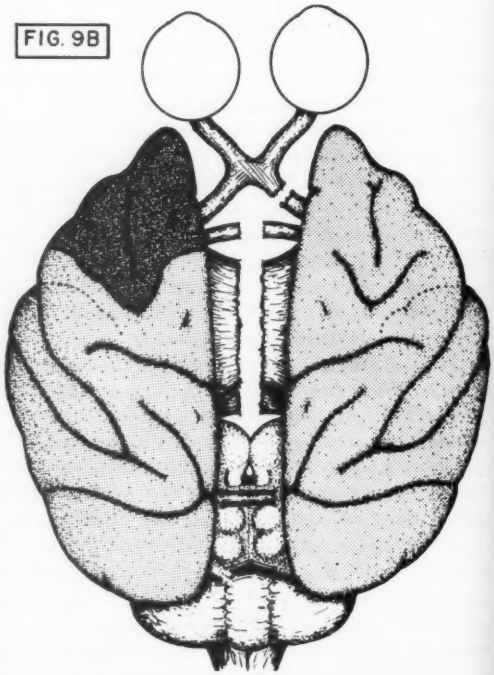


FIG. 7. Visuo-motor coordination and visual conditioning of right foreleg response survive this kind of surgical separation of visual and limb areas of cortex.

FIG. 8. Capacity to integrate visual stimuli with somesthetic stimuli from right hand survives surgical splitting and cortical removal as indicated.

FIG. 9. Two of various possible unilateral approaches to the classical temporal and prefrontal lobe syndromes.

Analagous to the above sensori-motor type of integration, there are various sensori-sensory integrations that can be analyzed to advantage with the split-brain technique. Figure 8 illustrates an approach to a form of visuo-tactile association. The monkey is trained to perform a discrimination problem that requires in each trial an association of both visual plus somesthetic cues. By controlling the hand and the eye used, and thereby the cortical receptor centers involved, we can test intra- and inter-hemispheric integration with and without different parts of the corpus callosum, and then with various types of separating cuts and ablations to analyze the kind of neural association required for such a performance.

It was somewhat of a surprise to find that with somesthetic stimuli entering the hemisphere opposite that of the visual inflow, the monkey was still able to perform the visuo-tactile integration. I first used color and weight (largely proprioceptive) discriminations (23) and have now repeated the results with black and white pattern and cutaneous rough-smooth stimuli. Performance with the latter is retained even after midline section of the habenular and posterior commissures, the massa intermedia, and the quadrigeminal plate in addition to the corpus callosum and anterior and hippocampal commissures. Removal of the bulk of somatosensory arm cortex, on the side of the visual input as indicated in the figure, abolishes the performance with the affected hand for several weeks. Meantime, however, the other hand continues to perform above 95% correct. This puzzling result is under further investigation.

In this connection I am reminded that it is perhaps as important as anything to point out that in working with this twin brain preparation we have often been forced into new patterns of thinking. That is, not only does one see new approaches to old problems, but in many cases one is forced into asking entirely new kinds of questions about brain physiology, questions of a sort that I, at least, would never even have thought of asking with the usual bilateral approach.

When we turn to the evidence regarding intermanual transfer of somesthetic discriminations and of motor learning in the primates, the results have not been entirely consistent (4, 6–8, 10, 15, 21, 23). In our own experience section of the cerebral commissures may lead to failure of intermanual transfer but not in all cases nor under all conditions. We have observed intermanual transfer in chiasm-callosum sectioned monkeys that were sophisticated in using either hand with either eye and were trained with pairs of objects that were left in sight because they were distinguishable only by touch not vision, the one being harder, or heavier, or looser than its mate and this being discernible only upon palpation. Also, removal of the somatosensory arm cortex as shown in figure 8 has been found to evoke transfer on subsequent testing in split-brain monkeys that had formerly failed to exhibit transfer on problems tested prior to the cortical removal (10). The interpretation of this is complicated at present by the fact that a trained reversal of the transferred task consistently fails to transfer back again to the first hand (10).

Some transfer effects also show up in visual learning, according to data obtained by Trevarthen in monkeys with presumably complete section of the callosum, anterior and hippocampal commissures and optic chiasm. So far the transfer influences seem to be confined to brightness, general color and possibly very simple forms of pattern discrimination. All these may be aspects of visual stimulation that cross at the collicular level of the midbrain and hence cases are being tested with more extensive midline sections that include the posterior commissure and quadrigeminal plate.

In figure 9*A* may be recognized a unilateral approach to the classical Klüver-Bucy temporal lobe syndrome and some of its subsequent fractionations (5) and in 9*B* the same for the old prefrontal lobe syndrome and its role in visual delayed response. In the former case the operation may make the monkey "unilaterally tame" for a while, i.e. placid in its response to touch and stroking of its limbs and body parts contralateral to the temporal lesion (John Steiner, unpublished observations). In the latter case (9) the delayed response performance is impaired in the absence of the usual hypermotility and distractibility that have followed bilateral removals and that for years, have tended to confuse the interpretation. In each instance one gains new data and information as to whether certain of the symptoms involve direct intrahemispheric relations or are perhaps more distant secondary effects stemming from hyperactivity, temperament changes, and the like, involved in the bilateral removals.

The so-called *encephale isolée*, and *cerveau isolé* preparations of Bremer and his group (1) have found considerable use in physiology. It should not be out of the question to go further and prepare isolated half-brains of different forms and degrees of isolation that would be used in the chronic condition, i.e. carried around by the animal *in vivo* in the brain's natural habitat under normal biochemical conditions, and recovered from the acute effects of diaschisis for use in implanted electrode conditioning and other physiological studies.

By combining various ablations and sections like those described above with local stereotaxie lesions in subcortical nuclei, it is possible today with methods now available to attain a fairly extensive surgical dissection of the mammalian brain, and to set up a large variety of combinations and permutations of cerebral centers in chronic preparations for functional testing and analysis. Combine with this the analytic possibilities brought by the chronically implanted electrode for recording, stimulating, and self-stimulating plus the new automated training and programming techniques, and those of us working in the physiology of behavior find ourselves today surrounded by seemingly endless new possibilities just waiting for the doing.

In the outlook for physiology, particularly that of the younger people, perhaps even those top level secrets of

brain function that to Professor Herrick were always primary and which gave a perspective and an added meaning to all his observations, whether he was looking at a synapse or at a hemisphere, need not remain so much longer, "impossibly remote and out of reach."

Our brain surgery on monkeys has been performed during the past 18 months almost exclusively by Dr. Harbans Arora. Lois MacBird has carried the major responsibility for the training, and medication of the animals and for general laboratory assistance. I wish also to thank Colwyn Trevarthen and Drs. Mitchell Glickstein and John Steiner for permission to mention some of their unpublished findings.

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"In attacking the problem of the origin and biological significance of the cerebral cortex comparative neurologists have hitherto generally directed their attention primarily to the cortex itself and its supposed primordia in the lowest vertebrates. As a result of these studies it is becoming increasingly evident that the key to this difficult question is to be sought in the subcortical centers of the primitive types, that is, in the 'old brain' (palaeencephalon of Edinger, segmental apparatus of Adolf Meyer), which attains its definitive pattern as a mechanism for reflexes and instincts prior in evolutionary history to the emergence of true cortex (neencephalon, or suprasegmental apparatus of the forebrain). It is important, therefore, that the exact pattern of these primitive sensori-motor mechanisms of the forebrain be determined in advance; for here are to be sought the primary physiological factors through whose interaction the functional pattern of the cerebral hemisphere has been elaborated." (C. Judson Herrick. The internal structure of the midbrain and thalamus of Necturus. *J. Comp. Neurol. 28:* 216, 1917.)

"The anatomical configuration and connections of the amygdaloid complex suggest that as a whole it possesses a certain physiological unity and that the component parts of this integrated complex are diversely represented in various vertebrate types, in accordance with their respective modes of life. The amphibian relations suggest, further, that the correlation of olfactory, gustatory, and perhaps other excitations arising from food within the mouth was the original integrating physiological factor. . . .

"The mammalian amygdala, like many other complex correlation centers, is a mechanism in which there converge into final common paths numerous very diverse kinds of peripheral excitation—some of visceral (interoceptive) type and some of somatic (exteroceptive) type." (C. Judson Herrick. The connections of the vomeronasal nerve, accessory olfactory bulb and amygdala in *Amphibia. J. Comp. Neurol. 33:* 274, 1921.)

Brain mechanisms and the learning process[1]

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IN PAYING HOMAGE TO THE MEMORY of Charles Judson Herrick, the neurophysiologist and neuroanatomist is confronted with a herculean task. For this man demands no prosaic elegy, no perfunctory appraisal of good research, neatly contrived and meticulously executed. Indubitably, all this was his, and yet there was so much more to the mighty frame in which he lived and worked. It is surely not too much to say that he has made for himself, in the truly epic quality of his research, a place amongst the immortals.

In the last 30 years, the neuroanatomist has found himself challenged on every hand by the physiologist's direct testing of virtually all of the major anatomical systems within the brain. This testing has cast into musty oblivion many of the classical anatomical concepts, and, at the same time, has revealed new systems and new patterns of organization which have been, in themselves, a challenge to the morphologist to seek new and finer ways of unravelling brain structure. Parenthetically, these recent joint physiological and anatomical studies have aimed, albeit with a slow and halting approach, at an understanding of the dynamic aspects of on-going patterns of organization in the living nervous system, insofar as they might relate to the brain's participation in behavioral processes.

It is in this very context that Herrick's singular genius showed itself. In his research, he stepped boldly from the morphological frame to postulates on the role of different brain regions in behavioral processes. He may be truly described as the apotheosis of a long line of such investigators, including Broca (15), Elliot Smith (19) and Johnston (34). Many aspects of his remarkable extrapolations and generalizations from the brain of the tiger salamander (29) have been tested in the finest of modern physiological and behavioral research and not found wanting.

Let us therefore take as our point of departure in this rather devious discussion of brain mechanisms in the learning process Herrick's (29) own evaluation of the development of the forebrain pallial areas in the salamander.

[1] The studies presented here were undertaken with assistance of Grants B-1883 and B-610 of the U. S. Public Health Service.

MORPHOLOGICAL CONSIDERATIONS IN FOREBRAIN DEVELOPMENT

Herrick (28) noted the differentiation of 3 pallial areas in the vertebrate forebrain. A dorsal pallium on the lateral aspect of the hemisphere lies between the hippocampal cortex on the medial wall and the pyriform cortex ventrally. In evolution, the reptiles were the first to show infiltration of this dorsal pallial cortex by non-olfactory fibers, which also spread to the adjacent hippocampal cortex, where a widespread intermingling of somatosensory and olfactory influxes takes place. Herrick further noted that the mammillary and interpeduncular nuclei might constitute final common paths from the olfactory cortical areas of the hippocampal and pyriform zones, and that the main motor outflow from these centers was directed into the apparatus of locomotion and mass movement.

It was to the interrelations of this so-called olfactory cortex with other cortical areas that Herrick's (28) attention was particularly directed. He remarked that, at all stages of cortical elaboration, an important function of the olfactory cortex, in addition to participation in its own specific cortical associations, is to serve as a non-specific activator for all cortical activities. He envisaged his generalized activity as primitive in type, acting on the neopallial cortex as a whole, "lowering its threshold, or increasing its sensitivity, or in the case of noxious stimuli, exciting an inhibitory influence. It gains expression in overt behavior, learning capacity and memory as a differential influence on exteroceptive sensorimotor mechanisms having well-defined anatomical and physiological localization." In the absence of any localization of its own, it may exert its effects either on exteroceptive systems producing external orientation, or upon the internal apparatus of general bodily attitude, disposition and affective tone.

RECENT STUDIES OF CORTICO-SUBCORTICAL INTERRELATIONS

It would seem apposite to mention here in historical purview the growth of ideas which have culminated in recent attempts to unravel the tangled skein of interrelations between the rhinencephalic cortex and subcortical

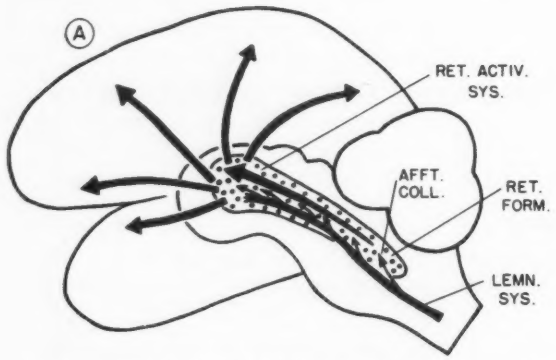
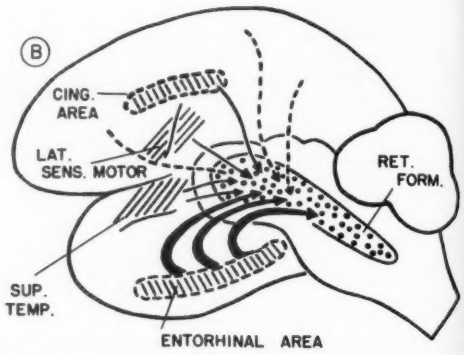


FIG. 1. Aspects of activity in the reticular activating system (*A*). Collaterals from principal sensory pathways may enter the ascending reticular system and so exercise widespread cortical influences.



Corticifugal influences (*B*), and particularly those from basa temporal areas, can modify conduction in ascending reticular systems. From W. R. Adey, *Australas. Ann. Med.*, 5: 153, 1956.

regions. Broca (15) first grouped these medial cortical areas under the term "limbic lobe." His studies have been reviewed by Maclean (40). In his "proposed mechanism of emotion," Papez (43) suggested a series of pathways from the hippocampal gyrus, through the hippocampus and into the fornix bundles to the mammillary bodies. Further relays in the mammillothalamic tracts carried this activity to the anterior thalamic nuclei, and so to the cingulate cortex, where it passed caudally through the cingulum bundles to once again enter the entorhinal and presubicular zones of the hippocampal gyrus.

This scheme of reentrant connections, and others which propose an essentially opposite series of intrahippocampal pathways, have been reviewed in detail elsewhere (3). It is not proposed to labor the intricacies of such schemes here, but it may be suggested that those based on the Papez model take little account of major influences exerted by hippocampal cortical structures at rostral midbrain levels, either independently of, or in addition to, any influence which these cortical areas may exert directly at the anterior diencephalic level in the perifornical zones and mammillary regions. Much anatomical, physiological and behavioral evidence has accumulated on the interrelationships of both amygdaloid and hippocampal parts of the limbic system with the reticular zones of the rostral midbrain (2, 5, 9–13, 21, 22, 24) in studies ranging from the marsupial to the primate.

Our studies have indicated that, in the marsupial and primate, these influences from the hippocampal gyrus are exerted in an essentially direct fashion through descending pathways running caudally from this cortex. Other investigators have proposed caudal extensions of the fornix system, either directly, or through the interposition of the septum, as the main pathway in the rat (27, 42). Certainly, the possibility of major species differences in the manner of interconnection between hippocampal and subcortical structures requires further investigation. The chronic stimulation studies of Hunsperger (33) have suggested that, in their participation in evoked affective responses, the perifornical zones of the anterior diencephalon and the rostral midbrain tegmentum are separate and partially autonomous entities, and cannot be construed as a continuum. This would support the point of view that hippocampal system may be set astride the brainstem and diencephalon at these two levels, rather than exercising its influences solely in the anterior diencephalon (9).

In a broader frame, brief reference may be made to participation of the hippocampal cortical system in the general scheme of ascending and descending reticular connections (fig. 1). Although the ascending reticular system (41) sends its influences widely over the cortical mantle from head-stations in the midline and reticular thalamic nuclei, its connections with the hippocampal cortex appear relatively indirect. The septum may be a way-station on ascending reticular pathways to the hippocampus. Evidence for this has been reviewed elsewhere (3), and it may be noted that such a scheme of hippocampal influxes was clearly proposed by Elliot Smith (19) and later by Herrick (28). These hippocampal influxes from the septum may reach the hippocampal pyramidal cell layer after relaying in the dentate granule cells. Evidence from septal stimulation supports the view that such a caudally directed scheme of pathways from anterior diencephalic areas can activate not only the hippocampus, but also the subjacent cortex of the hippocampal gyrus (entorhinal area). This cortex, in turn, projects to midbrain reticular areas and is capable of profoundly modifying ascending reticular conduction (11) to a greater degree than corticifugal influences from other parts of the cerebral mantle (fig. 1). Many widespread connections with caudal diencephalic and rostral midbrain areas have been disclosed anatomically after temporal lobectomy (16). Their place in the scheme of physiological interrelations is at present uncertain.

THE ROLE OF TEMPORAL LOBE STRUCTURES IN THE LEARNING PROCESS

It is some 20 years since Klüver and Bucy (35) described in dramatic detail the behavior changes following

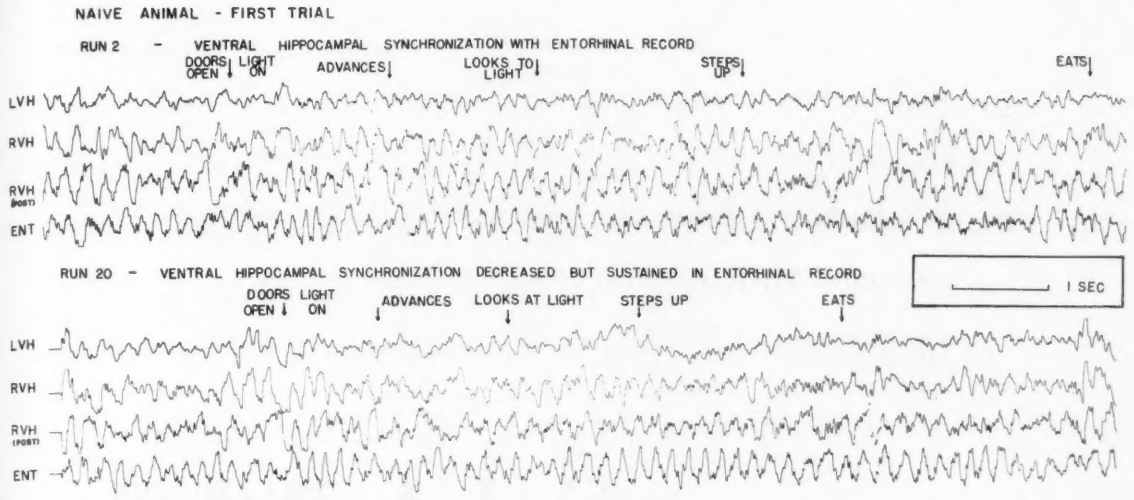


FIG. 2. Modifications in hippocampal records in the course of initial approaches in T-maze box. Initially, ventral hippocampal (*LVH, RVH*) and entorhinal records (*ENT*) are synchronized, but by *run 20* (lower record) only the entorhinal lead shows sustained rhythmic discharge during approach. From Adey *et al.* (8).

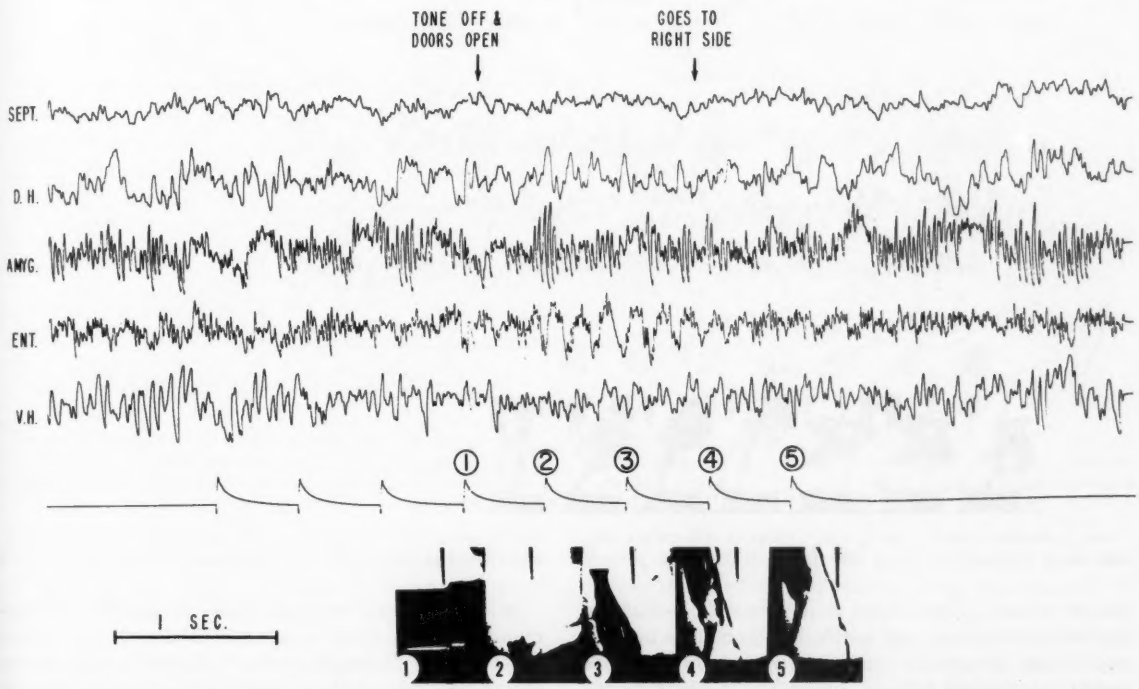


FIG. 3. Typical 6 cps burst in entorhinal lead (*ENT*) during approach to food in T-maze box. Numbers on EEG record correlate with photo frames below. At (*1*) start-box doors are closed, open at (*2*) and cat starts correct approach to left side of T-box, completed in frame (*4*). Other abbreviations: *AMYG.*, amygdala; *D.H.*, dorsal hippocampus; *V.H.*, ventral hippocampus; *SEPT.*, septum. From Adey *et al.* (8).

bilateral temporal lobectomy in the monkey, which they succinctly characterized in the term "psychic blindness." As an invariant concomitant of this condition, there is a loss of recent memory, and in the years that have followed the description of this Klüver-Bucy syndrome, a similar and gravely incapacitating interference with recent memory and the learning process has been described in man following interference with the anterior deep temporal regions (45).

It is obvious that testing of the role of the hippocampal

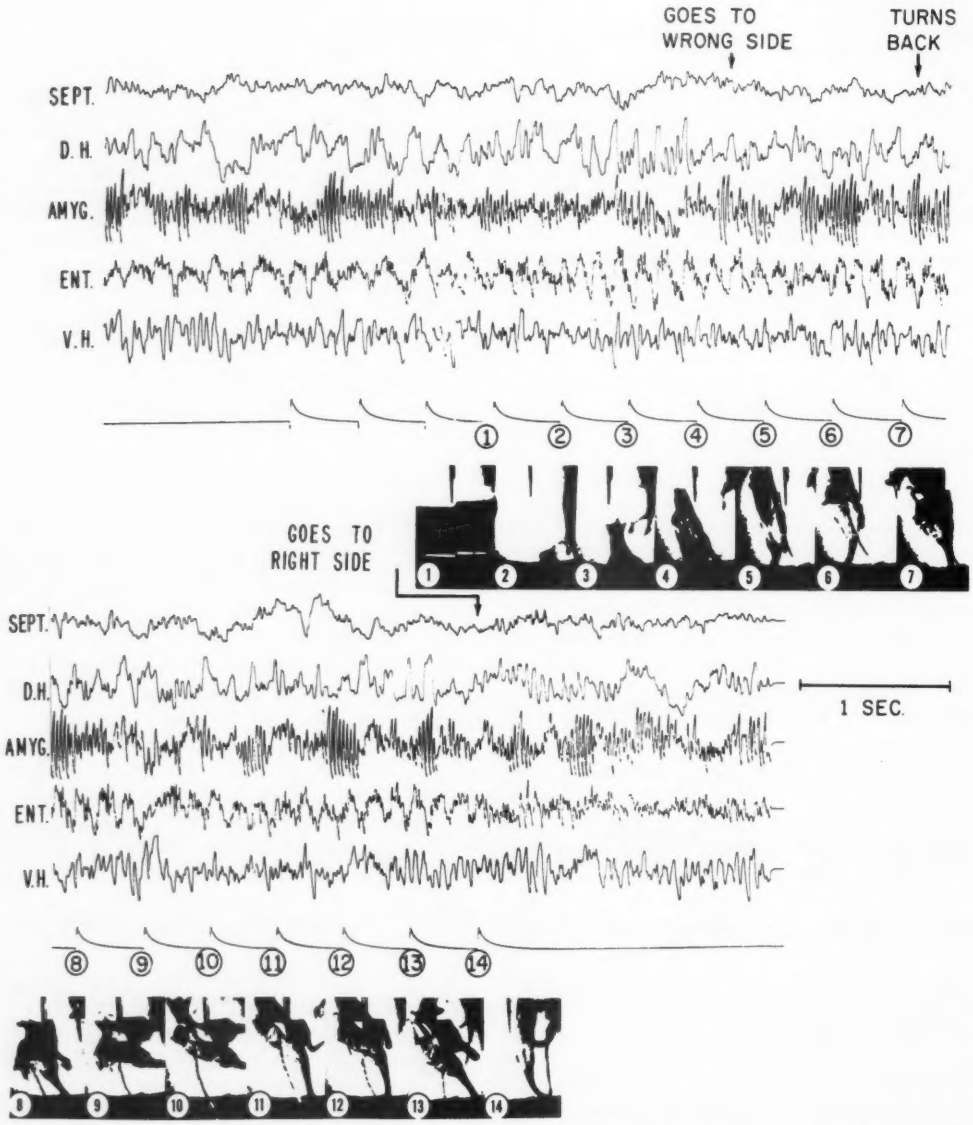


FIG. 4. Similar record to fig. 3, with 3 episodes of 6 cps activity, first during approach to wrong side (*frames 2–5*), then as it turns back towards start box (*frames 6–9*), and finally corrects its approach to right side (*frames 12–14*). From Adey *et al.* (8).

system in such a behavioral context is only possible in the alert, responding, and preferably, freely moving animal. Using chronically implanted electrodes, we have sought correlations between hippocampal electrical activity and certain learned performances (4, 8, 31, 32).

Correlates of hippocampal electrical activity in T-maze learning. Green and Arduini (25) observed regular trains of slow-waves in the electrical activity of the rabbit's hippocampus during states of alerting and emotional arousal. They noted that the onset of these wave-trains with a sudden stimulus coincided with the disappearance of the rhythmic waves in the neo-cortical records of the conscious resting animal.

With electrodes implanted in pyramidal cell and dentate granule cell layers of both dorsal and ventral parts of the cat's hippocampus, as well as in the adjacent entorhinal zone of the hippocampal gyrus, and in the amygdaloid complex (fig. 5), we have examined the distribution of slow-wave activity in the course of T-maze training (8). Over 40 animals have been trained to approach a food reward in a T-box, on the basis of a visual cue. Ten seconds were allowed for the approach, and the animal was rewarded only if the initial approach was directly to the correct side of the box.

Particular attention has been directed to the changes in hippocampal activity occurring in the early phases of

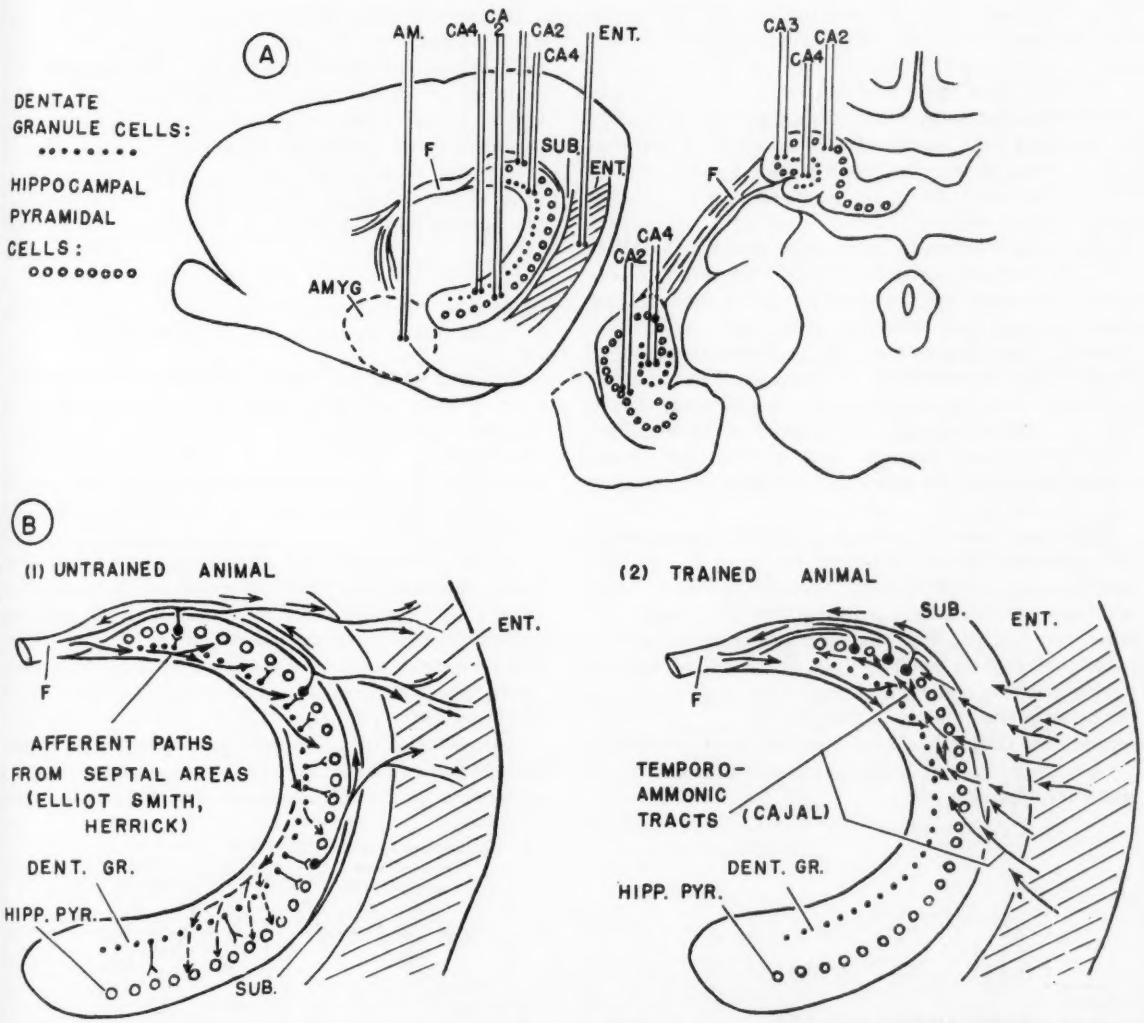


FIG. 5. *A*. Placement of implanted electrodes in regions CA_2, CA_3 and CA_4 of dorsal hippocampus and in similar zones of ventral hippocampus. *B*. Suggested schemes of physiologically active pathways in early and late training, on the basis of computed phase relations. Abbreviations: *AMYG*, amygdala; *DENT. GR.*, dentate granule cells; *ENT.*, entorhinal area; *HIPP. PYR.*, hippocampal pyramidal cell layer; *F.*, fornix; *SUB.*, subiculum. From Adey *et al.* (8).

training. In the naive animal, on first placement in the test box (fig. 2), rhythmic trains of slow-waves were found in the period before any reward was presented. At this time, these rhythmic trains were synchronized in widely separated zones of the dorsal and ventral hippocampus, and covered almost an octave of frequencies between 4 and 7 cycles/second.

Major changes took place in both distribution and frequencies of the slow-waves in the course of the first 10 to 20 presentations of the approach situation. Rhythmic activity rapidly declined in ventral hippocampal areas during successive approaches, and did not reappear in these areas in the course of subsequent training to a high level of performance (fig. 2). By contrast, a regular slow-wave discharge persisted in dorsal hippocampal areas and in the entorhinal cortex (fig. 2). This rhythmic discharge occurred throughout training, and showed no adaptation even at high performance levels. Autocorrelation computing of the frequencies involved (see below) has indicated that this discharge during the approach performance is in a narrow frequency spectrum between 5.5 and 6.5 cycles/second.

Detailed examination of this 6 cycles/second activity in the entorhinal area (*ENT*, fig. 3) has shown that the burst begins with a latency of 300–500 msec after the presentation of the situation, and lasts through the period of the approach performance. In a further example (fig. 4), it will be seen that there are three clear bursts of this slow-wave activity, the first associated with an approach to the wrong side, the second as the animal

turns back and moves towards the starting end of the box, and the third with a final approach to the correct side.

On the basis of these findings, which were extremely consistent in the many animals trained, and the data to be presented from studies of delayed response performance, it is suggested that this rhythmic 6 cycles/second burst is associated with a goal-directed performance. In recent careful studies, Grastyan *et al.* (23) have correlated certain aspects of hippocampal slow-wave activity with the Pavlovian orienting reflex. This reflex, as defined by Konorski (36), involves turning of the head and trunk towards the stimulus rather than locomotion towards a goal. Our records (fig. 4) show no evidence of this 6 cycles/second activity at times when the animal's head and trunk were undergoing turning from side to side in a fashion strongly reminiscent of classical Pavlovian orientation, although such activity was present during locomotion towards a goal immediately preceding and following periods of orientation.

Hippocampal slow-wave activity in delayed response training and extinction. In this situation, the animal saw food placed under one of two cans 7 seconds before a bridge was lowered, which then permitted the cat to approach either can (31, 32). Records from the hippocampus and entorhinal area in animals at a high level of training showed varying amounts of slow-wave activity during the delay period, mainly in the spectrum 3.5–4.5 cycles/second, and distinctly slower than the burst of waves at 5.0–6.0 cycles/second associated with either correct or incorrect approach.

During extinction of the response, no burst of slow-waves followed the lowering of the bridge in the habituated animal, although activity at 4–5 cycles/second still occurred in the delay period. Characteristic 6 cycles/second bursts rapidly reappeared during the approach performance on retraining. Similar extinction studies in cats in the T-maze box have also indicated a replacement of the 5–6 cycles/second activity characteristic of the approach performance by slower 4 cycles/second waves.

COMPUTING OF AUTO- AND CROSS-CORRELATION FUNCTIONS OF WAVE BURSTS IN EARLY AND LATE TRAINING

Data reduction techniques, which, in many instances, have already had wide application in the analysis of complex wave processes in the physical sciences, have been used in recent years to provide some objective measure of aspects of inherent rhythmicity, and to look for possible hidden rhythms not revealed by inspection of EEG records.

In our studies described above, auto- and cross-correlation functions have been computed along the lines of Brazier and Barlow (14). In the absence of more sophisticated recording equipment, many of our paper-written records have been digitized by hand on punch-cards, and the correlation functions computed on an IBM 709 digital computer (8).

Auto-correlations of records from both dorsal hippocampal and entorhinal regions showed a strong tendency towards a sinusoidal waveform during the approach

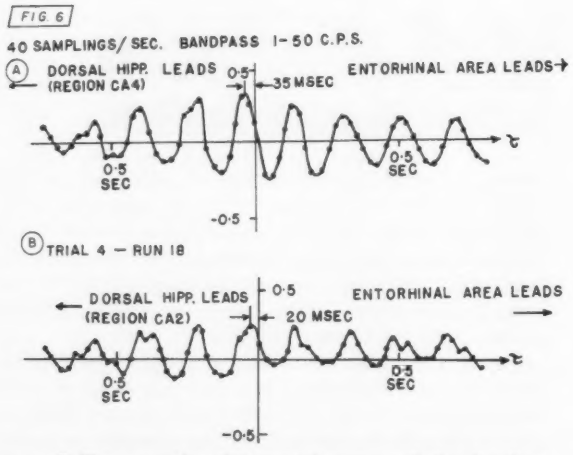


FIG. 6. Two examples of computed cross-correlation functions between the entorhinal area and two different regions of the dorsal hippocampus (*cat 7, trial 4, runs 14* and *17*). In each case, the dorsal hippocampal zone leads the entorhinal area by 20–35 msec. From Adey *et al.* (8).

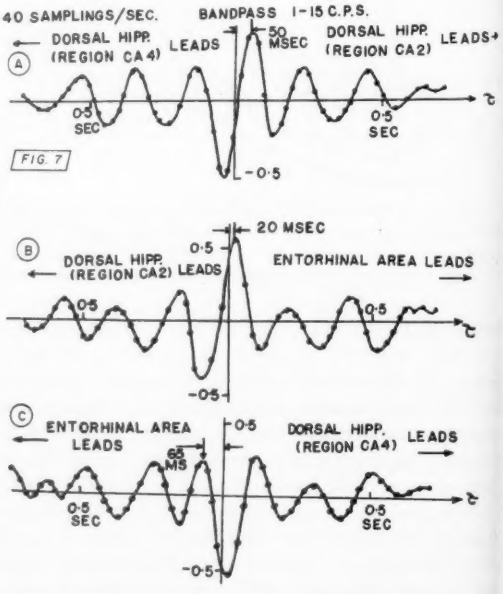


FIG. 7. Cross-correlations of records from same leads as in fig. 6 in late training (*cat 7, trial 20, run 4*). Entorhinal area now leads hippocampal zones by 20–65 msec. From Adey *et al.* (8).

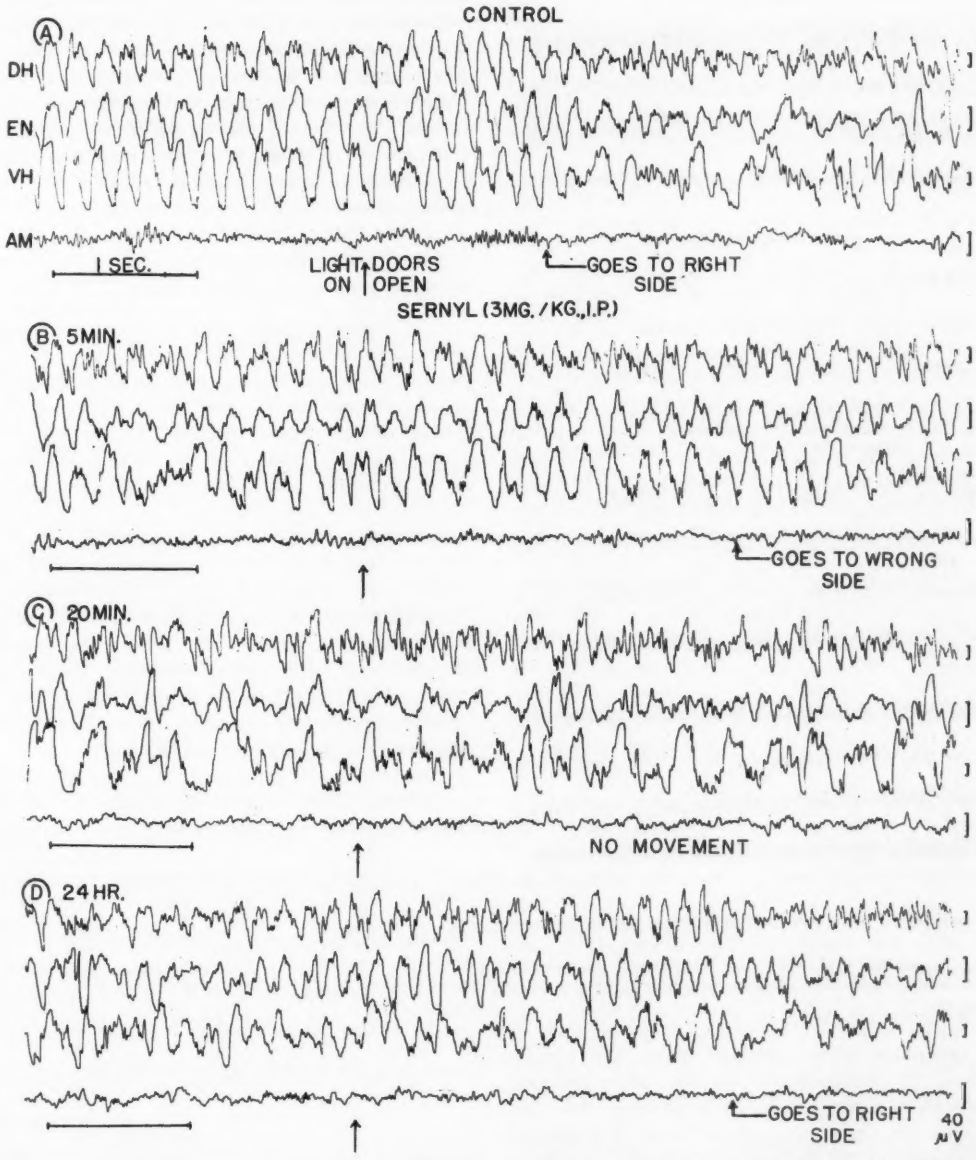


FIG. 8. Effects of a psychotomimetic cyclohexamine derivative, Cl-395, on cat's performance in T-maze box. Failure of performance was correlated with decline in 6 cps burst during approach. Lead abbreviations: *AM.*, amygdala; *D.H.*, dorsal hippocampus; *EN*, entorhinal area; *V.H.*, ventral hippocampus. From Adey and Dunlop (7).

performance. By contrast with this strongly rhythmic 6 cycles/second burst activity during the approach, autocorrelations of the ' background" or resting activity between each approach have shown a strong periodicity at 3–4 cycles/second.

Cross-correlation of one channel with another has allowed an assessment of the constancy of phase angles between bursts from different parts of the hippocampal arch at various stages of training. In early training (fig. 6) cross-correlations have consistently shown that dorsal hippocampal records from both hippocampal pyramidal cell and dentate granule cell zones (regions CA_2 and CA_4 of Lorente de Nó (38), respectively) lead the entorhinal record by 20–35 msec. In striking contrast, these phase relationships in late training in the same animal were entirely reversed (fig. 7), with the entorhinal area leading the dorsal hippocampal bursts by as much as 65 msec. Further application of these computing techniques now in progress has indicated certain consistent differences in phase relationships in records from an animal at a high performance level between correct and incorrect decisions.

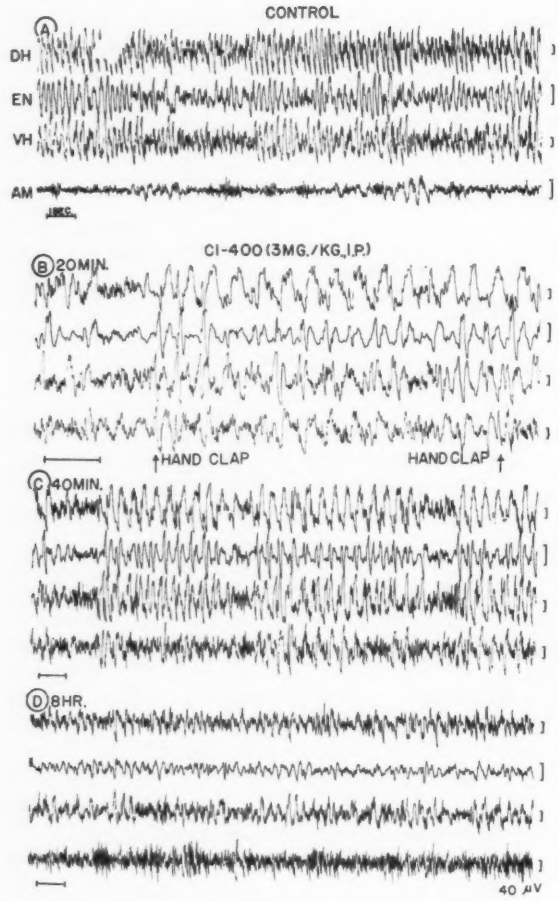


FIG. 9. Seizure activity induced by Cl-400, particularly in entorhinal (*E.N*) and ventral hippocampal leads (*VH*), and exacerbated by sudden stimuli (*B* and *C*). Normal rhythmicity had not returned 8 hours after drug instrumentation. From Adey and Dunlop (7).

These computed phase patterns may, with certain reservations, be extrapolated to the morphological systems shown in figure 5*B*. In early training, the computed results are consistent with a series of pathways from the vicinity of the dentate granule cells in CA_4 to the hippocampal pyramidal cells in CA_2, and thence to the entorhinal area. Such a scheme of hippocampal influxes bears a strong resemblance to that proposed by Elliot Smith (19) and Herrick (28), with septal and anterior thalamic areas providing the main afferent hippocampal activity and with further relays to the entorhinal area as suggested by Lorente de Nó (38).

The trained animal shows, by contrast, a pattern of phase relations consistent with activation of the temporo-ammonic tracts arising in the entorhinal area, as proposed by Cajal (17). The terminal ramifications of these tracts are found in relation with the dendritic trees of the hippocampal pyramidal cells, and the short axons of the dentate granule cells similarly terminate in these dendritic arborizations. In the trained animal, activity adjacent to the dentate granule cells lags behind that in the hippocampal pyramids by about 50 msec, and by 50–70 msec on activity in the entorhinal area. Convergence of dentate axons and temporo-ammonic tracts on the dendrites of the hippocampal pyramids might thus provide a structural basis for a phase-comparator mechanism in the pyramidal cell dendrites to be discussed below.

EFFECTS OF PSYCHOTOMIMETIC AGENTS ON LEARNED PERFORMANCE AND CORRELATED EEG RECORDS

The recent introduction of certain cyclohexamine derivatives in clinical surgery has indicated that these agents have psychotomimetic qualities in addition to their analgesic action (39). Patients have reported a variety of experiences, ranging from feelings of unreality to sensations of dissolution and death. Disorientation is often profound, and may persist over several days with doses as small as 0.1 mg/kg.

We have tested two of these agents, 1-(phenylcyclohexyl) piperidine monohydrochloride (Cl-395, Sernyl) and the other n-ethyl-1-phenylcyclohexylamine monohydrochloride (Cl-400, cyclohexamine), on the learned performance of cats in the T-maze box, and on the simultaneous EEG records from the hippocampal system (7). In doses between 1 and 3 mg/kg, these agents produced consistent electrical and behavioral changes in animals at a high level of performance.

Control records prior to drug dosage (fig. 8*A*) in such an animal showed typical 4–5 cycles/second activity in the waiting period, and a burst at 6.5 cycles/second

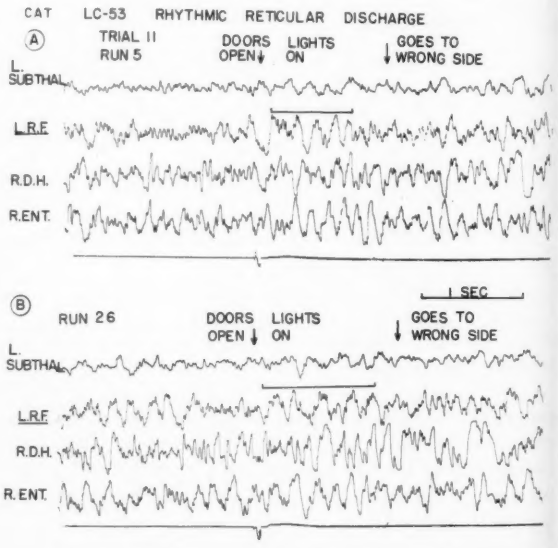


FIG. 10. Examples of rhythmic reticular discharges (indicated by *bar*) concurrently with rhythmic bursts in the hippocampal system during approach in T-box. Abbreviations: *L.R.F.*. left rostral midbrain tegmentum; *L. SUBTHAL.*, left subthalamus; *R.D.H.*, right dorsal hippocampus; *R. ENT.*, right entorhinal area.

during the approach. Five minutes after dosage with Cl-395 (3 mg/kg), there was a marked decline in rhythmicity of the record, with no 6 cycles/second burst during the approach, which was to the wrong side (fig. 8*B*). Twenty minutes after drug dosage, the cat was unable to perform the learned task, and remained crouched in the start box. At this time, records from the hippocampus and entorhinal area showed irregular slow-waves and scattered spikes in the entorhinal cortex (fig. 8*C*). Twenty-four hours later, when the response level had recovered to 70 per cent correct, approach responses were associated with the typical 6 cycles/second burst, although the "background" hippocampal activity in the waiting period showed less than control levels of rhythmicity, and the response latency was greatly lengthened (fig. 8*C*).

Examination of the records in the period of total failure of performance (fig. 9) has shown much seizure activity, with trains of seizure spikes in the hippocampus frequently initiated by sudden peripheral stimuli, such as a hand-clap (fig. 9*B*). This spiking was frequently maximal in the entorhinal cortex (fig. 9*C*). Normal rhythmicity had not returned after 8 hours (fig. 9*D*) and there were periodic exacerbations of seizure activity for 16–24 hours. At these times, the cat's performance of the learned task fell to a low level.

SLOW-WAVE ACTIVITY DURING APPROACH IN EXTRA-HIPPOCAMPAL STRUCTURES

The foregoing drug studies have indicated a possible correlation between disruption of normal patterns of hippocampal activity and the ability to perform a learned task. Penfield (44) has concluded from his surgical studies in man that "without the hippocampus and the hippocampal gyrus, the recording of current experience is impossible."

It is here that we must face one of the most fundamental problems in our notions of brain mechanisms in the learning process. As Penfield points out, "we must tread warily, for we do not know whether the ganglionic record of the stream of consciousness is located there, or whether the hippocampal structures play an essential part in the laying down of the record elsewhere." The following data indicate at least the probability that aspects of a common rhythmic process are to be found during the approach in both hippocampal and extra-hippocampal structures. With my colleague D. O. Walter, we have sought evidence of rhythmic processes phase-locked to the hippocampal discharges during the approach in subthalamic and midbrain reticular areas, and also in the primary visual cortex. It was found that such rhythmic discharges occurred frequently in the rostral midbrain reticular areas (fig. 10). They usually

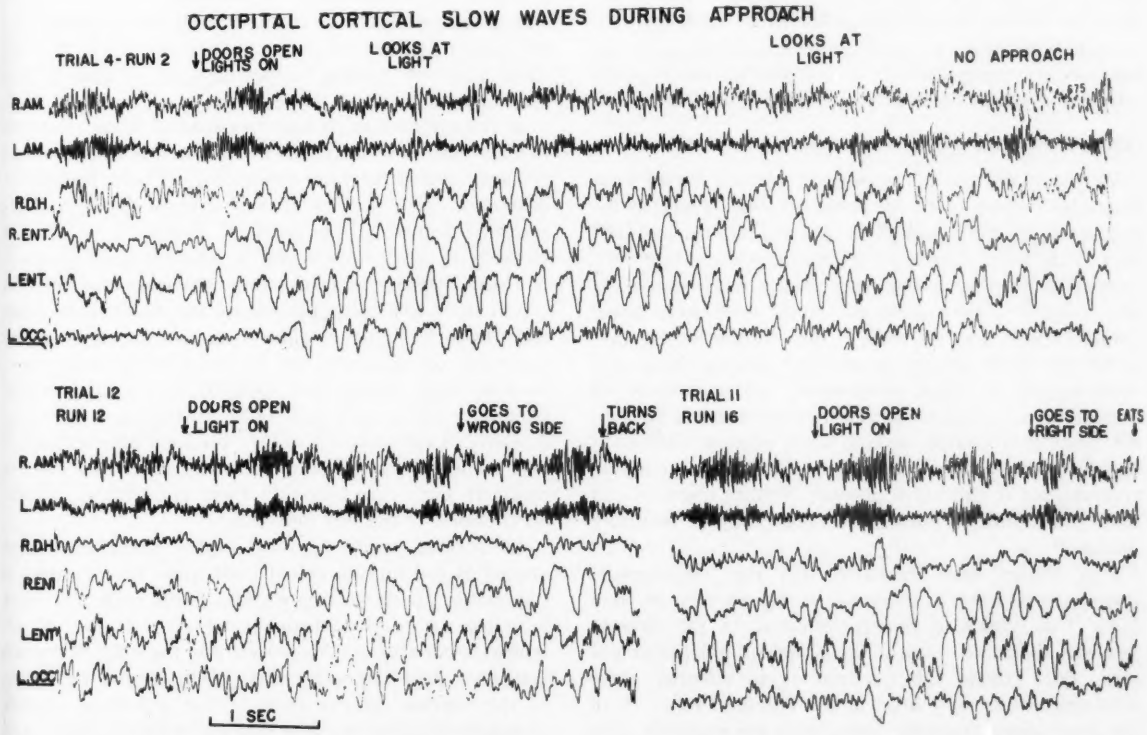


FIG. 11. Appearance of rhythmic slow-waves in primary visual cortex (*L. OCC.*) concurrently with hippocampal (*R.D.H.*) and entorhinal (*L. ENT.* and *R. ENT*) 6 cps activity. This occipital cortical activity became more clearly frequency-locked to the hippocampal discharges in late training (lower records).

coincided in onset with the rhythmic hippocampal discharge, and were most clearly phase-locked to the hippocampal discharge at onset. Later cycles of the reticular discharge frequently lagged or led the hippocampal slow-waves. Similar discharges were recorded in subthalamic areas, but were less obvious than in rostral midbrain reticular formation.

Finally, records from the primary visual cortex have indicated a similar incidence of phase-locked discharges (fig. 11). In early training (top record, *L. OCC.*), irregular slow-waves occurred during the approach, with only one or two cycles clearly phase-related to the entorhinal discharges. In later training, at a 70–80 per cent performance level, these occipital slow-wave discharges regularized and were much more clearly phase-related to the entorhinal slow-waves over many cycles of the burst (lower two records). There was a strong tendency for slow-waves in the hippocampal system to persist beyond the end of the occipital cortical burst.

These data thus suggest a physiological basis by which the hippocampal system may indeed exert an influence on both non-specific ascending systems in the midbrain reticular formation and also on activity in the primary receptor areas of the visual cortex. Future research may be expected to tell whether such rhythmic activity impinging on subcortical centers, and through them, on primary receptor areas in the cerebral cortex, may be essential to the process of the laying down of a memory trace, or to its recall at appropriate times. This is surely a fascinating prospect, even though it would bring us but one tiny step further in the long journey to an understanding of the brain's role in behavioral mechanisms.

INTERPRETATIONS AND CONCLUSIONS

We have skirted in this survey, without direct confrontation, many of the problems of conveyance of information in the brain. Our preoccupation here has been with the rhythmic wave process, without reference to unit activity occurring concurrently in the same tissue. Logically, two questions would arise from these considerations. Firstly, what is the relationship, if any, of the unit firing pattern to the wave process? Secondly, what aspects of neural integration, and specifically, of informational transfer, can occur between nerve cells on the basis of a graded, analog wave process, without a digital, or pulse-coded output as a necessary or inevitable consequence of this wave process? Neither question can receive a definitive answer, but both may be fruitfully discussed.

Our studies have indicated that the hippocampal slow-wave activity is maximal in the vicinity of zone CA_4. This region is closely adjacent to the densely grouped dendritic trees of the hippocampal pyramidal cells, thus raising the question as to whether these rhythmic waves may arise in the ephaptic relations of the interwoven dendritic trees, with the initiation of a propagated cellular discharge as a minor, rather than a causal feature of the rhythmic process. Certainly, the simultaneous records of unit activity in the hippocampus (26) and the entorhinal area (2) do not suggest that the cellular spike discharge always occurs in a constant relationship to successive cycles of the slow-waves, or that cell firing, though briefly accelerated, is maximal in the course of the slow-wave discharge. Supporting evidence comes from studies of cell firing in the cortical alpha bursts (37), and also in the cellular behavior in hippocampal (20) and cerebellar seizures (18), where rhythmic seizure waves have been recorded in dendritic cortical layers in the apparent absence of cellular firing. A dissociation of cell firing from the wave process has also been noted in the globus pallidus (6).

The data presented here would suggest the possibility of significant integration occurring in dendritic mechanisms, and in associated presynaptic terminals, without propagated cellular discharges as an inherent and inevitable consequence of these integrative processes. The subtle shifts in patterns of phase relations in the slow-waves described here in the course of approach training have suggested the possibility of integration on the basis of a phase-comparator mechanism which would conceivably operate at the cellular level, and involve graded analog processes (8). Such a concept is not proposed as one to replace aspects of pulse-coded informational transfer in the brain, since obviously transmission to distant centers must certainly be based on such a scheme. It is rather that such a concept would hopefully draw attention to the possibility of informational integration and, perhaps, its storage temporarily or permanently, on the basis of physico-chemical changes induced by graded wave processes passing in specific spatio-temporal patterns across sheets of nerve cells.

In this connection, it may be useful to draw attention to what may be termed learning and non-learning neural systems, and to certain morphological and functional distinctions that may be drawn between them. A characteristic feature of the cerebral ganglia or brains of virtually all invertebrates and vertebrates is the presence of a sheet-like or corticoid arrangement of nerve cells, in which there is ample opportunity for dendritic overlap and the direct influence of local, graded processes in one nerve cell on adjacent cells. Even the lowly earthworm, with no more than a few hundred cells in its cerebral ganglia, exhibits such a palisade arrangement (1). The majority of such systems so far examined have exhibited "spontaneous", wavelike electrical activity, and show a similarly high correlation in their capacity to exhibit conditioned or learned behavior.

In striking contrast, such highly complex neural organs as the human spinal cord must be regarded as essentially non-learning systems. Even with the most judicious use of physiologically appropriate stimuli, attempts to condition reflex behavior in the isolated human cord have proved unsuccessful. Attention may be drawn to the absence in the cord of the structural criteria characterizing the cerebral systems outlined above, and to the absence of a concomitant wave process as a major aspect of its electrical activity. Extrapolations from such systems as the spinal cord to the physiological processes

of cerebral systems in learning would seem of dubious value.

As a signpost to the future, and in the humble awareness of the magnitude of the problem, we might, perhaps, direct our examination of behavior and its mechanisms to those external and intrinsic factors which influence its patterns of manifestation and provide its motivating power. As Herrick (30) has remarked, "all behavior is a transaction between the organism and the field within which it lives. Most behavior is directive, the end toward which it is directed being the satisfaction of the motivating conditions. The study of motivation must include both the conscious factors and the unconscious processes which precede and accompany them."

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Nervous mechanisms of behavior

C. JUDSON HERRICK
1868–1960

THE HONOR AWARDED ME BY THIS CONFERENCE is gratefully acknowledged, because in the twilight of one's day such expressions of regard by his colleagues are the highest rewards of scientific endeavor.

It is with trepidation that I venture to open this discussion with some comments on the nervous control of behavior, for it must be admitted that a neuroanatomist's contributions can at best be heuristic. He can only suggest favorable places and methods and tentative hypotheses for experimental testing. Comparison of observed structure with observed behavior may justify inferences that point the way toward profitable fields for further study. We have seen many instances of experimental verification of such inferences and some notable failures.

It has been my privilege to see the development of comparative neurology as an organized science almost from its beginning. This experience has led to the formulation of some general principles of the mechanics of behavior, as elaborated in my book of 1956 on *The Evolution of Human Nature*. Here I shall mention three of these principles, without the evidence or details of their operation.

These are: First, properly controlled observation of introspective experience is as valid and useful a scientific method as is reflexology. Second, the distinction between analytic and integrative processes and mechanisms is fundamental and its recognition clarifies much that has been obscure. Third, the neuropil is the mother tissue from which the specialized central functional systems have been derived in vertebrate phylogeny, and it plays an important role in learning.

CONSCIOUS MOTIVATION

All behavior is motivated by something either extrinsic or intrinsic to the behaving body. The distinction between genetic and personally acquired factors of behavior is fundamental, although often hard to recognize. In general, the genetic factors are more stable and are common (with minor variations) to all members of a race or species, as typified by reflexes and instincts.

Among the intrinsic factors, it is incontestable that, in the human domain, conscious motivation, including awareness of the end desired and of the means available for its satisfaction, is in some instances a significant factor in determining the pattern of behavior.

If and when we ever find out just how these conscious factors fit into the total scheme of cerebral organization, we shall not have explained them away. The hoary mind-body problem will never by solved by ignoring the troublesome factors. The way conscious motivation actually works in human society and its relation to more primitive unconscious factors I have examined in an article entitled A Biologist Looks at the Profit Motive (*Social Forces*, 16: 320–327, 1938).

More than a decade later I wrote an introduction to Nina Bull's book on *The Attitude Theory of Emotion* (New York: Nervous and Mental Disease Monographs, 1951) in which the mechanisms involved in Mrs. Bull's theory are briefly considered. In this and many other papers she has given experimental evidence of the way conscious and unconscious factors interplay in the motivation of emotional behavior. She applies feedback and other acceptable principles in original ways that open inviting fields for further study.

During the last 50 years we have seen revolutions in both physics and psychology, yet it is still true that we are today as ignorant of the basic principles of psychophysics as primitive man was ignorant of the physical principles of mirage, with frequent resort to mysticism in both instances. We do not yet know enough about those operations of the brain that have as one of their properties some awareness of what is going on to be able to formulate a plausible hypothesis of the mechanics of thinking. It does no good to postulate a mystic anima that lives in the body but may on occasion move out. Any species of dualism is a confession of defeat, and defeatism is not a healthy attitude in science. What we need is some still unknown system of mechanics (perhaps nonquantitative in nature) that differs as radically from known systems as quantum mechanics differs from Newtonian mechanics.

ANALYSIS AND INTEGRATION

It has repeatedly been emphasized that the factors of motivation concerned with adjustments to the external environment differ radically from those, like homeostasis and many others, that regulate internal operations of the body. The former may be characterized for short as 'analytic,' the latter as 'integrative.' This difference

is basic in all animals, although all factors previously mentioned are intricately interrelated in all behavior.

The various organs of sense and the related nervous elements are analyzers in Pavlov's sense, and the peripheral motor nerves are similar analyzers, selecting from all possible patterns of response the one that is appropriate for the sensory information received. This selection is made by the central integrating apparatus. The peripheral and central analytic functions are typically stimulus-response reactions. Their organs are stable patterns of nervous connections that are similar in basic design in all vertebrates, however much they may differ in detail. They have been described in many animals, which is fortunate because integration can operate only on information supplied by them.

Our knowledge of the intimate structure and mechanics of operation of the integrative apparatus is still pitifully incomplete, but it is adequate to show that in both of these respects it differs fundamentally from the analyzers.

The analytic functions are rigorously regulated in adjustment to the spatial and temporal relations of things and events, with the behaving body and the moment of its action as fixed points of reference. Integration, on the contrary, is a much more labile process. It is a total pattern of behavior as Coghill defined it, and the spatial and temporal factors are more relativistic in the broad meaning of this term. In some acts of integration space and time may be mutually interconvertible, as illustrated by the way the temporal series of auditory vibrations sensed by the ear is registered in a spatial series of loci in the cerebral cortex and again transformed into a still different dimension when recognized subjectively as pitch of the tone. Many other instances have been described. In the higher domains of cortical integration, such as the fabrication of concepts of truth and fidelity, there may be no spatial or temporal factors at all. This supports the suspicion that the mechanics of such operations may involve some nonquantitative patterns that cannot be expressed numerically.

Both the analytic and the integrative apparatus are progressively elaborated and complicated as we pass from primitive to higher vertebrates, the latter far more than the former. The analytic systems reach their culmination in the human cortical projection areas, the integrative systems in the intervening areas of memory and elaboration.

In motivation genetic and acquired factors and both analytic and integrative processes may be involved in numberless different ways. In the process of learning all these factors may play an ancillary part, but its distinctive features are personally acquired integrative acts.

There is no consensus about how learning is to be defined. If we say that basically it is conditioning of reflexes, we know that this ability has been observed and measured in species as low in the scale as flatworms. It is probable that primordial protoplasm has some similar ability to change the pattern of behavior by personal experience. This has been shown to be true even in plants.

From this it follows that we must sharply distinguish learning from intelligence and learning cannot be used as a measure of intelligence. Much can be learned with no increment of intelligent behavior, however the latter is defined. It is what one does with his learning that counts. I have known some very learned men who were surprisingly unintelligent.

MECHANISMS OF LEARNING

When I began to study comparative neurology seventy years ago I devoted about ten years to mapping the functional components of the cranial nerves of fishes. When I tried to extend this analysis into the higher levels of the brain I found the tissue so complicated that accurate tracing of the functional systems was impossible with methods then available. I then examined the brains of salamanders and their still more primitive allies, hoping to find that here the central connections of the basic reflex systems were so simplified as to be more easily followed.

This hope was soon shattered. To my surprise I found the peripheral sensory nerves, with their clearly segregated systems of functional components, upon entering the brain branched widely with innumerable terminal ramifications in an intricate tangle of thin unmyelinated fibers from various sources. In this neuropil the fibers of several different modalities of sense are entangled. From it fibers pass to the motor nuclei from which arise the peripheral fibers that again are clearly segregated according to their functions. The sensory pathways from this neuropil to higher centers do not transmit impulses of any specific modality, but those which result from the interplay of activities within the neuropil, where every contact is a potential synapse.

The difference between this primitive structure and that of the higher vertebrates, with their well defined primary nuclei and secondary tracts for the several modalities of sense, is explained by the fact that most activities of salamanders are mass movements that do not require the refined analysis of sensory and motor systems of the higher vertebrates.

This closely meshed neuropil is spread everywhere throughout the brain of the salamander in both the white and the gray substance. It evidently is the mother tissue from which the refinements of both analytic and integrative apparatus have been differentiated in the course of phylogeny, with more precise and stable localization of function in the first and preservation of more labile properties in the second.

We do not know how the sensory analyzers are able to inform the motor nervous apparatus what pattern of response is appropriate. There is increasing evidence (notably that given by Paul Weiss and R. W. Sperry) of a chemical specificity of the several functional systems of nerve fibers. Some of these are innate and some are acquired in later life. How this works out in the learning process may be illustrated by a brief reference to Hebb's conception of assemblies of functionally inte-

grated nervous elements. Each assembly is so constituted chemically or otherwise that its excitation can trigger other assemblies of similar constitution that are in attunement with it.

A first experience of any kind activates several assemblies of the first and higher orders which interact with one another. They may be genetically so constituted that the response follows automatically as reflex or instinct. If not, there may be trial and error fumbling until some new experience triggers the appropriate motor assembly. This satisfactory outcome is in some unknown way imprinted on the structure so that upon repetition of this experience the appropriate response is facilitated. This is memory, and successive repetitions establish a habit. Something has been learned.

As specialization increases in the higher animals, the assemblies become more specific and diversified, but their elements may not be condensed in a single locus. Most such assemblies probably include neurons in widely separated parts of the brain. Mammalian learning must include a hierarchy of assemblies with very complicated interconnections.

The human brain is so organized that the highest assemblies may interact among themselves without participation of those of lower orders. A problem that long study has failed to solve may establish tensions that are resolved by subconscious interplay of these higher assemblies, resulting in a flash of "insight." One may go to sleep at night vexed by failure and in the morning waken with the solution of the problem brilliantly clear. Our ability to use language and other types of symbols both rationally and unconsciously, thus economizing muscular work by expenditure of a smaller amount of cortical work, is largely responsible for all cultural progress.

The integrative functions are, of course, localized while in process, but this localization is not determined by the fixed innate structural pattern of the tissue. It fluctuates from moment to moment, and similar functions may be performed at some other time and place wherever the requisite kind of tissue is available.

Failure to recognize the radical difference between analytic and integrative functions is responsible for the prevailing confusion about the principles of cortical localization of function. The projection areas, including many only recently discovered, are fairly stable representatives of the highest levels of analysis. The rest of the cortex has ill-defined areas that are obviously preferential for different kinds of elaboration. But an acquired function, such as some specific instance of learning, is not necessarily performed in the preferential area, as clearly shown by our clinical and neuropathological knowledge of the aphasias.

C. Judson Herrick

All parts of the mammalian brain except the masses of myelinated fibers that form the white matter are permeated by an undifferentiated neuropil that ties the assemblies together. Which system of assemblies is activated in any situation is determined in part by the internal excitatory state—such influences as fatigue, the chemical composition of the body fluids, and many others.

From this it follows that no human experience is ever exactly repeated. If any assembly is temporarily or permanently incapacitated, some similar assembly may be triggered to give an appropriate response. This again involves learning.

This kind of learning by substitution of an impaired system of assemblies by some other system that activates a different but serviceable motor response is minimal in subhuman vertebrates, as Sperry has shown; but the human brain is so organized that this is possible, as proved by the spectacular achievements of current programs for rehabilitation of the handicapped.

"The democratic ideal has never been fully realized. The peoples of the earth have tragically failed to find a satisfactory answer to the basic problem of how to live together harmoniously and productively. At the present moment progress is obstructed by a recrudescence of nationalistic ideologies and lust for personal and group supremacy by methods that are essentially bestial rather than human. The most powerful of these methods are not reversions to bestiality, for they use refinements of cruelty of which no beast is capable. . . .

"The patterns of human social organization—the best of them as well as the worst of them—are deeply rooted in the biological origins of human nature. The primordial 'primitive altruism' has

no ethical implications whatever. Our most urgent task now is to preserve this biological heritage and to refine and strengthen it with moral sanctions. . . .

"The fundamental issues here are not those of power politics. They are essentially moral. The moral issues evidently have biological significance as such and so they can be evaluated and implemented scientifically. Under existing conditions moral values are survival values and we ignore them at our peril." (C. JUDSON HERRICK. *The Evolution of Human Nature*. Austin, Univ. of Texas Press, 1956, pp. 186–187.)

". . . We have at our command all the physical and mental resources that are needed for further advance [toward more efficient mental techniques for widening the range of experience and rational interpretations of it] . . . if only we do not squander them in senseless and suicidal rivalry and conflict. . . .

". . . The democratic unit cannot function or long survive unless the individual selves first learn to govern themselves and to sacrifice some personal profits and preferences for the welfare of the group. This is altruism. Quite apart from any ethical considerations and in terms of strictly biological survival value, altruistic behavior marks the highest level of cultural evolution. It is a key factor, and the only one that can relieve the tensions that now endanger all the cultures of the world. This is not a speculative vagary or a fantasy of wishful thinking. It is validated by the whole history of human evolution and especially clearly by current events." (C. JUDSON HERRICK. *The Evolution of Human Nature*. Austin, Univ. of Texas Press, 1956, pp. 220–221.)

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RECENT ADVANCES IN THE BIOCHEMISTRY OF VIRUSES[1]

Introductory remarks

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THE SYMPOSIUM to be presented this afternoon was designed by your officers to acquaint you with some of the more exciting and significant contributions of biochemistry to our understanding of viruses.

Our conception of what viruses are and how they reproduce has altered considerably in the past 20 years. It is possible, today, to think about viruses in biochemical terms. They are no longer mysterious microorganisms, characterized by small size, failure to propagate in cell-free media, and ability to cause disease.

Viruses are ubiquitous organisms, and have been shown to infect or parasitize plants, insects, animals, and even bacteria.

Much of our present knowledge is the result of the extensive and brilliant investigations of a number of people on bacterial viruses. The importance of the bacteriophage system is reflected by the fact that three of the five papers in this symposium deal with these organisms. The plant viruses have also been extensively investigated, and the pioneer efforts of Stanley on the physicochemical properties of viruses were done with the tobacco mosaic virus. Recently, techniques have become available that allow us to obtain comparable information about the animal viruses, as will be illustrated by the papers of Doctors Colter and Ginsberg.

It has become increasingly evident that viruses vary greatly in regard to their physicochemical and biologic properties; however, certain generalizations can be stated that would seem to apply to them all.

Mature virus particles are composed of nucleic acid, either ribonucleic or deoxyribonucleic acid (RNA or DNA), which are surrounded by, and probably complexed with, a coating of protein that also may contain lipid and polysaccharide. The coat would appear to have the function of protecting the nucleic acid when it is released from the cell and to assist it in gaining entry into new host cells. It may be a relatively simple structure, such as in the tobacco mosaic virus, in which it is a series of "doughnuts," piled up to form the characteristic hollow rod. On the other hand, it may be highly complex, as exemplified by certain of the bacteriophages. The nucleic acid of these organisms is contained within the head, a shell of protein that is attached to a rodlike tailpiece, through which the DNA is introduced into the cell. Within the tail are incorporated contractile elements, enzymes for digesting a hole into the bacterial cell wall, and other components. Altogether, a most remarkable biologic apparatus that has been evolved, in nature, to transport a bit of DNA from one bacterium to another.

The nucleic acid of the virus particle gains access, through the mediation of its "coat," into the interior of the cell. Here, it induces the cell to reproduce not only more viral nucleic acid, but the various components of the "coat" as well. The complete particle is assembled and released to infect other cells, and the cycle repeated. The release of virus may be associated with lysis of the cell or may proceed for varying periods without apparently compromising the economy of the cell. The latter situation would seem to obtain in infection with tumor viruses. The primary role of the nucleic acid in transmitting to the cell the information required for viral replication has been well established by the demonstration that, under the proper conditions, nucleic acid alone, extracted from a number of plant and animal viruses, can produce infection resulting in the production of complete virus.

The principal information incorporated into viral nucleic acid relates to production of more virus. However, the nucleic acid may remain present in the cell and be reproduced with each cell division, but no complete virus is manufactured, unless induced by manipulation such as exposure to ultraviolet light. In bacteriophage infection, this phenomenon is referred to as lysogeny. Of great interest is the observation that, under the conditions of lysogeny, genetic attributes that appear to have nothing to do with replication of virus may be conferred upon the cell. An example of such a situation is that of the diphtheria bacilli, which produce toxin when lysogenized, but not otherwise. A possible analogy is provided by virus-induced malignancies. As a result of

[1] A symposium held in Chicago, Ill., April 15, 1960. Sponsored by the American Society of Biological Chemists.

infection with the virus, the cells acquire those genetic attributes that distinguish them from normal cells. Not only may viral infection confer upon the cell new properties, but the genetic material of the cell in which the virus was grown may become so intimately associated with the virus that genetic characters of the original host cell are introduced along with the virus particles into the newly infected cells (transduction).

It should also be pointed out that, like all other biologic organisms, viruses mutate. These changes in genetic properties undoubtedly represent changes in the nucleic acid moiety which, in turn, influence the composition of the viral "coat."

It is evident that viruses present the investigator with a form of genetic material somewhat simpler and more readily available for biochemical and other studies than that of a whole cell. It is to be hoped that—from studies of the fundamental properties and behavior of viruses—will come information that will allow for the treatment and control of viral infections and, perhaps, cancer on rational, rather than empiric, bases.

However, aside from these important and potentially practical considerations, viruses provide excellent subjects for the study of the biochemical aspects of genetics and protein synthesis, and for probing the biochemical anatomy of the cell.

Thus, the geneticist, the virologist, and the cell physiologist find their investigations overlapping more at the fundamental level. Their common ground is, to a large extent, biochemistry particularly that of the nucleic acids.

Genetic control of protein structure in bacteriophages

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ANY DISCUSSION of the genetic control of protein synthesis in viruses must, at the moment, necessarily be more concerned with posing questions than with answering them. My own discussion of the subject, as part of this program, will not deal so much with viruses as objects of study for their own sake as it will, rather, with the use of viral systems as convenient tools for the study of the important biological question of "coding." The question of the relationships between the genetics of an organism and the structure of its phenotypic proteins is a central one, and one to which the protein chemist can contribute.

Everyone is aware of the generalized scheme summarized in Fig. 1. We see, at the top of the figure, an idealized portion of the DNA double helix, which we presume to be the major source of information that controls the structure and general physiology of any given organism. The scheme, occasionally referred to as the "central dogma," proposes that information, coded in this structure in a linear fashion, is translated into protein structure by metabolic processes that are, at the moment, unknown but which, very likely, involve RNA. This scheme is essentially a rephrasing of the well known Beadle-Tatum hypothesis, which states that there exists a one-to-one relationship between individual gene and individual enzyme (1). Solving the genetic code, in terms of this drawing, means demonstrating that specific changes in the molecular structure of DNA are reflected in corresponding changes in the polypeptide chain in the center of the drawing.

In its simplest form, the transference of information from gene to protein would involve only the translation of a linearly arranged base-pair message into a linear amino acid sequence. It is clear, however, that most proteins are not simple polypeptide chains, but are folded and cross-linked to yield three-dimensional, globular molecules. Furthermore, every protein molecule of a given variety appears to be the same, within the limits of experimental measurement, as every other molecule in the sample. To attain such uniformity, there must be, in the genetic "code," either ancillary bits of hereditary information that determine secondary folding, tertiary interactions, and covalent crosslinking, or else sufficient information in the amino acid sequence itself to predetermine the proper coiling and stabilization of the polypeptide chain.

Although it is much too early to attempt to predict which of these alternatives is correct, experience gained from the study of the reoxidation of the ribonuclease chain (2–4), after complete reduction of the four disulfide bridges present in the native protein, suggests that the arrangement of charged groups and of various lyophobic and lyophilic side-chains may be sufficient to direct the subsequent formation of a standard, three-dimensional protein structure. The last step in the scheme shown in Fig. 1 may, therefore, be independent of special genetic surveillance.

The term "gene" has, of course, a number of meanings, since it is a completely operational term, and its definition depends on the way in which it is studied. In the sense that it was used by Beadle and Tatum, the gene is defined as a genetic unit of enzymatic function and, on the basis of classical crossing-over techniques, would have a size of the order of 1,000 A. The radiation geneticist, on the other hand, might define the size of a gene as that portion of chemical material within which a "hit" by a proton or electron would knock out the function in question—he might suggest a much smaller size.

A most elegant and revealing approach to the study of genes by genetic methods has been of the kind made by Benzer (5) and others who have shown that there exists, within any given functional genetic unit, a very large number of point loci, each of which must be intact for proper function of the unit. Benzer has coined the term "cistron" for such a unit, and has shown that a single cistron may contain hundreds of distinguishable subunits.

The problem that I would like to consider deals with experimental work, now in progress in many laboratories, aimed toward the elucidation of the chemical relationship between individual cistrons and the protein structures whose synthesis they control. We already know that such a relationship must exist, from the work that has been done on the abnormal hemoglobins, first, by Pauling and Itano and their colleagues (6, 7) and, subsequently, by Ingram (8) and by Hill and Schwartz

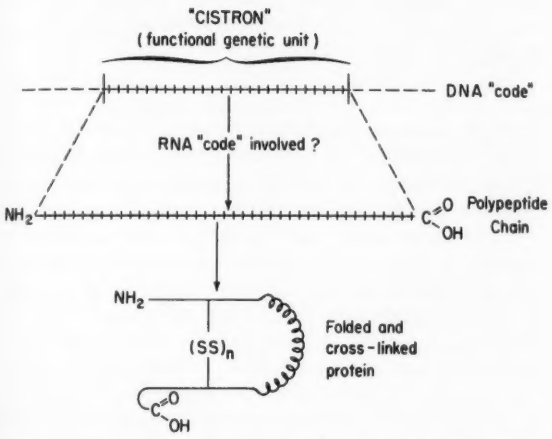

FIG. 1. Postulated steps in the transfer and utilization of genetic information during the synthesis of a protein chain. As discussed in the text, the last step of the process may not require genetic control beyond that coded into the amino acid sequence of the fully extended polypeptide chain.

(9). These studies have shown that single mutations in the genetic material controlling the biosynthesis of hemoglobin cause unit changes in the amino acid sequence of one or the other of the hemoglobin chains. It is unfortunate, however, that a detailed examination of the problem cannot be made efficiently with the human hemoglobins, because of the great difficulties involved in attaining sufficiently finely structured genetic maps with human material. For this reason, most of the effort being expended on the decoding problem is being applied to bacterial and viral systems. Viruses and, particularly, bacteriophages are ideal test objects for such work, since generation times are short and the yields of progeny high. Thus, a very large number of events can be observed and scored with good statistical accuracy.

Much of the work on the genetic control of protein structure in viruses is being done with the T-even bacteriophages. Fig. 2 shows an electronmicroscopic picture of a bacteriophage particle taken by Brenner and his colleagues at Cambridge University (10). The viral suspension was pretreated with peroxide, which causes minor changes in the structure of the particle. A number of clearly recognized structural elements are visible in this photograph, including the "head," which acts as the protein envelope for the DNA of the phage particle; the tail fibers, which determine the host-range of the strain; the tail "core," through which is ejected the DNA strand after attachment of the virus to its host bacterial cell; and the tail sheath, in this case contracted as the result of peroxide treatment.

Fig. 3 shows a schematic reconstruction of the structure of a phage particle. I have indicated, at the bottom of the drawing, the location of one protein component that does not appear, at least obviously, in the electronmicroscopic pictures, namely lysozyme. Three of the structural components of the bacteriophage particle have so far been subjected to the study of changes induced by mutation. The first component, the tail fibers, has proved to be extremely recalcitrant from a chemical standpoint, although an elegant genetic map has been constructed for the host-range cistron by Streisinger and Franklin (11). As shown in Fig. 4, it was possible to make partially purified concentrates of the h-protein by column chromatography of urea-solubilized bacteriophage ghosts (12). The protein in question could be detected in the effluent fractions from the column by an immunological technique. It has also been possible to prepare highly purified tail fibers by column chromatography of phage preparations dissociated by low pH and digested with trypsin, work that has been carried out by the group of investigators at the Cavendish Laboratory in Cambridge, England (10). However, the protein proved to be so large—about 100,000/subunit—and the "fingerprints" so complex (10, 13) that the proper study of its structure was not attempted.

Brenner and his colleagues have also investigated the protein of a second component, the bacteriophage head, which makes up the bulk of the proteinaceous material

FIG. 2. Electron microscope photograph of a bacteriophage particle. The phage preparation was pretreated with peroxide, causing the contraction of the tail sheath. From Brenner *et al.* (10).

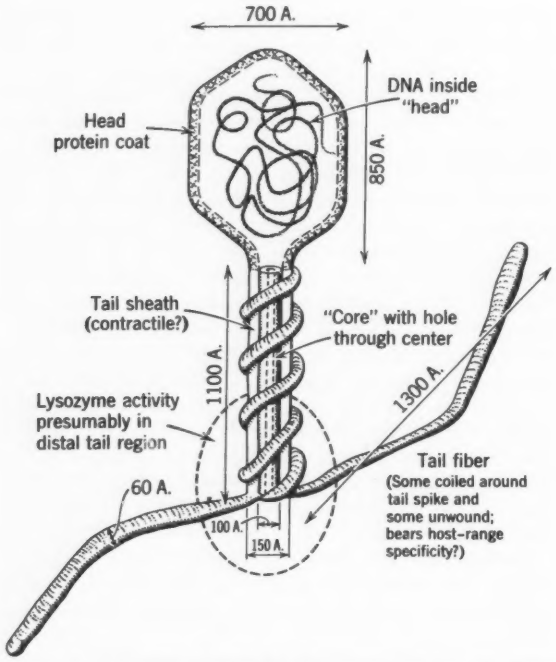

FIG. 3. Schematic drawing of a bacteriophage particle. From Anfinsen, C. B., *The Molecular Basis of Evolution*. New York: Wiley, 1959, p. 77.

of the virus. They have been able to construct a map for the genetic region that controls the structure of this component, employing, in their genetic analyses, variations in the sensitivity of the virus to osmotic shock. The Cambridge group is, unfortunately, also plagued with the large size of this protein component, and may have technical difficulty, therefore, in relating changes in sequence to mutational loci.

The third protein component of the bacteriophage particle that has been examined chemically and genetically is the enzyme, lysozyme, whose possible location in the virus particle is indicated schematically in Fig. 3. Before discussing this protein in more detail, let us first examine the generalities of the chemical approach to the "information-transfer" problem. The problem of relating genetics to protein structure obviously consists in two major operations: the first consists in the selection of mutant forms that differ from the parent organism by a single genetic change, and the arrangement of a number of such point mutations in a linear genetic map; the second, in isolation of the corresponding protein from each of the mutants and structural comparison of the mutant proteins with the original "standard-type" protein molecule. Since a large number of modified proteins must, in theory, be compared, it is essential that simple, yet fairly precise, methods be employed for partial sequential analysis. The method of choice, at the moment, is the technique commonly known as "fingerprinting" or, perhaps more descriptively, "peptide-mapping." The highly purified protein is digested with a proteolytic enzyme, generally trypsin because of its high degree of specificity for lysyl and arginyl peptide bonds. Digestions are performed either on the native protein, if it is susceptible, or on the protein, after chemical oxidation or reduction of disulfide bridges. Ideally, one obtains, with trypsin, a number of fragments equal to one more than the total number of lysine-plus-arginine residues. The peptide mixture may be separated by successive two-dimensional paper chromatography and

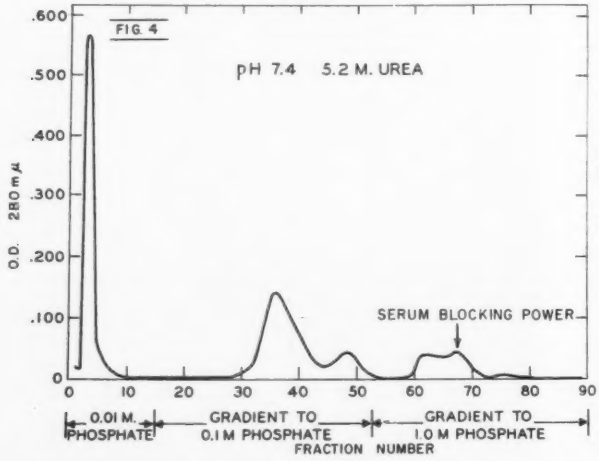

FIG. 4. Chromatography of T_2 bacteriophage proteins on XE-64. Partial purification of the protein component of bacteriophage T_2 involved in the determination of host range ((13) and unpublished experiments, W. D. Dreyer, A. Katz, and C. B. Anfinsen). From Anfinsen, C. B., *The Molecular Basis of Evolution*. New York: Wiley, 1959, p. 182.

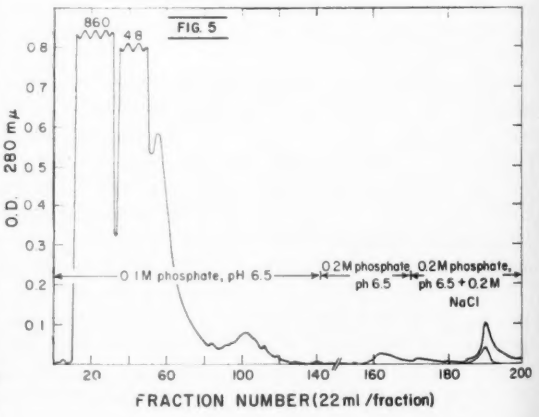

FIG. 5. Isolation of phage lysozyme; XE-64 column chromatogram. Partial purification of lysozyme from bacteriophage T_2 (15). Lysozyme activity (*bottom curve*) is present only in the last component emerging from the chromatographic column at approximately fraction #190.

TABLE 1. *Amino Acid Analyses of Lysozyme From Bacteriophage T4D*

	Residues per Mole (*ca. 15,000 mol wt.*)			
Lys	(18.6, 17.2)	18–19	Gly (10.0, 11.1)	11
His	(1.0, 1.0)	1	Ala (19.9, 21.2)	21–22
Arg	(8.0, 8.0)	8	Cys/2 ?	(0–1)
Asp	(16.5, 17.7)	18	Val (9.1, 9.3)	9–10
Met	(2.2, 2.4)	2–3	Ileu (8.2, 8.1)	8
Thr	(8.0, 9.2)	9–10	Leu (11.7, 12.7)	13
Ser	(4.9, 6.4)	6–7	Tyr	2–3
Glu	(13.8, 14.7)	15	Phe (5.6, 6.2)	6
Pro	(3.4, 2.9)	3	Trypt	1–2
			Total residues	152–161

paper electrophoresis (13) and, with scrupulous care and the avoidance of contamination with salt and of other materials that might influence the method, one can obtain highly reproducible and dependable peptide patterns with relative ease. There is insufficient time for proper description of the mapping technique; I will, therefore, only illustrate by presenting some typical results that have been obtained on the phage lysozyme system. These studies are part of a continuing collaboration between W. J. Dreyer and G. Streisinger, with M. Crumpton, F. Mukai, G. Koch, and myself contributing to various aspects of the work.

Koch and Dreyer (14) showed, about two years ago, that the lytic enzyme associated with the T-even bacteriophages was a lysozyme that appears to split the same bonds in *E. coli* cell walls as the enzyme in chicken-egg white. The enzyme is completely absent in non-infected *E. coli* cells, but is produced in excess, over that required for progeny phage synthesis, on infection of the host cell with the virus. Phage-induced lysates of *E. coli* contain on the order of 1–2 mg of the enzyme per liter. With ion-exchange resins, Dreyer and Crumpton (15) have been able to isolate pure samples of phage lysozyme, and have characterized the protein by a variety of physical methods. The first step of the procedure is shown in Fig. 5. The enzyme has a molecular weight of approximately 14,500, as determined by the Archibald equilibrium-sedimentation method. Table 1 summarizes a set of preliminary analyses of the amino acid composition of the enzyme after thorough purification on columns of carboxymethyl cellulose. The two values for each amino acid represent analytical figures obtained for 48-hour and 72-hour acid hydrolysates, respectively. The most probable value for each amino acid residue is given in terms of the number of residues per 15,000 molecular weight. The enzyme has 18–19 lysine residues and 8 arginine residues and should, consequently, yield approximately 28 peptide fragments on digestion with trypsin. It probably contains only one residue of tryptophane and either no cysteine or one residue. It would appear very likely that this protein consists of a single polypeptide chain devoid of disulfide cross-linkages with, perhaps, one sulfhydryl group. It is, at any rate, a very fortunately constructed protein, from the standpoint of sequential analysis.

Now, although lysozyme was absent from uninfected *E. coli* cells, it was still possible that the synthesis of the enzyme was under the control of *E. coli* genetics rather than phage genetics and that its synthesis might be induced by low molecular weight materials introduced into the host cell during the process of invasion. To decide this question, lysozyme was isolated from lysates of *E. coli* cells that had been infected either with T2, T4, or T6 phages. Fig. 6 shows a comparison of the peptide patterns obtained from purified T2 and T4 lysozymes (15). The patterns have shown reproducible differences in a number of experiments. The majority of the corresponding peptides, as well as those which differed, have been eluted from such peptide maps and analyzed qualitatively for amino acid composition. Table 2 shows the result of a few of these analyses. At the top of the table are listed some of the peptides that were found to be common to both T2 and T4 lysozymes. The lower part of the table shows analyses of two peptides, one of which is present in T4 and one in T2. Further differences were observed in patterns of T6 bacteriophage lysozyme, and it seems quite certain that the structure of the enzyme is, indeed, under the control of the information coded into the DNA of the virus particle. The main purpose of the preliminary studies of the bacteriophage lysozyme was, of course, to furnish a basis for the subsequent study of lysozyme from various mutant phages all derived from the same "standard" parent strain.

Mutant T4 bacteriophages, producing lysozymes different from that of the standard strain, can be recognized by a change in the lysozyme-produced "halo" that surrounds treated phage plaques. Plaques produced on agar plates containing a suspension of *E. coli* strain B are exposed to chloroform vapors (Fig. 7), a treatment that renders host cells susceptible to lysis by free lysozyme (15, 16). Standard-type phage plaques become surrounded by pronounced haloes, and rare mutant plaques lack, or have very small, haloes. These haloes reflect the presence of lysozyme that has diffused outward from the plaques during successive rounds of infection and lysis. That the mutations really affect the lysozyme molecule itself is made likely by the fact that lysozyme (either crude or purified) produced by mutant phage is more temperature-sensitive than that produced by the standard virus (16, 17).

Fig. 8 shows typical peptide maps obtained for the parent T4D lysozyme and for that of a heat-labile mutant, here labeled e4 (16, 17). Peptide mixtures subjected to the separation technique were prepared by trypsin digestion, for various lengths of time, of lysozymes that had been either heat-denatured prior to digestion or that had been first treated with mercaptoethanol in 10 M urea solution to reductively cleave any possible disulfide bridges in the preparations. Free sulfhydryl groups were then converted to the S-carboxymethyl derivative by

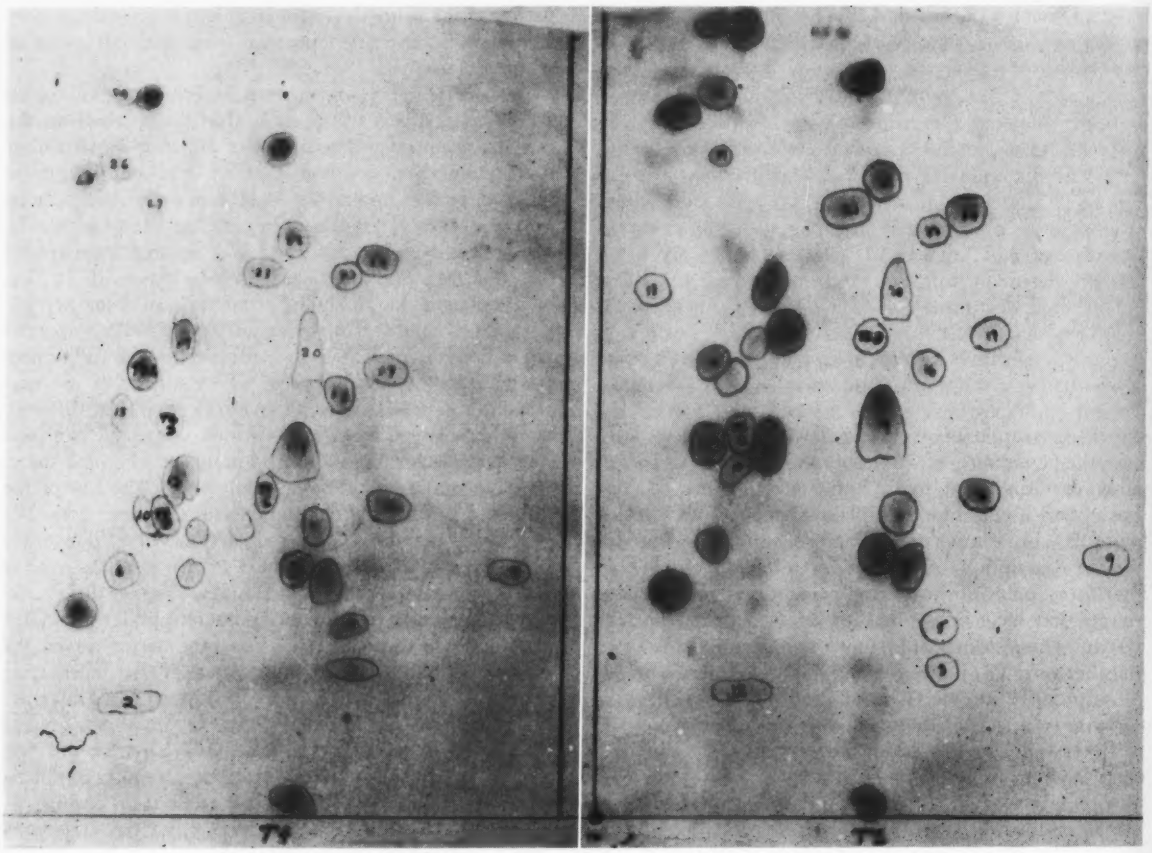

FIG. 6. Peptide maps of lysozymes from T_2 and T_4 bacteriophages grown on *E. coli B*. As described in the text, these maps are prepared by successive chromatography and electrophoresis of trypsin digests of the reduced-alkylated or heat-denatured proteins.

TABLE 2. *Partial Comparison of Trypsin Fragments of Lysozymes from Bacteriophages T2 and T4*

A. Partial List of Peptides Common to Both T2 and T4

(Asp, Glu_2, Ala) Lys
Leu(or Ileu) . Lys
Ala . Lys
(Leu [or Ileu], Gly, Ala) Arg
(Leu [or Ileu], Tyr) Lys
(Ala_{2-3}, Thr, Pro, Ser, Gly, Asp, Val_2, Tyr, Try, Phe, Leu) Lys

B. Differences

T4A ———— (Ser, Ala, $Glu(NH_2)$ Leu) Lys
T2A (Ser_2, Ala, Pro, Val, Leu) Lys ————

reaction with iodoacetic acid. The conclusion, reached on the basis of the amino acid analyses mentioned above, that phage lysozyme contains no disulfide bridges was strengthened by the observation that the peptide patterns were the same whether heat-denaturation or reduction-alkylation was employed prior to digestion. The "fingerprints," illustrated in Fig. 8, show only one reproducible difference (heavily encircled or cross-hatched in the center of the pattern). The encircled peptide component at the bottom, right, of the pattern for e4 does not represent a variation from the "standard" pattern but, rather, furnishes an excellent example of the sorts of pitfall that "fingerprinting" can involve. In this case, the peptide, which contains methionine, has been lost in the digest of the T4D enzyme, presumably due to oxidation of the methionine sulfur. Clear-cut differences and similarities can only be established when they are obtained reproducibly and on independent digests prepared from different protein samples.

As such data accumulate, it will be of very great interest to compare the location of changes in the amino acid sequence with the structure-function relationships of the enzyme. One expects to find, of course, that mutations will not be detected in those regions of the sequence that are critical for function or perhaps in those regions that have very little functional significance. Such mutations would either be lethal or would cause no obvious change in phenotype, and might, therefore, pass unobserved. In spite of the temporary excitement invoked by the detection of a covalent change in protein

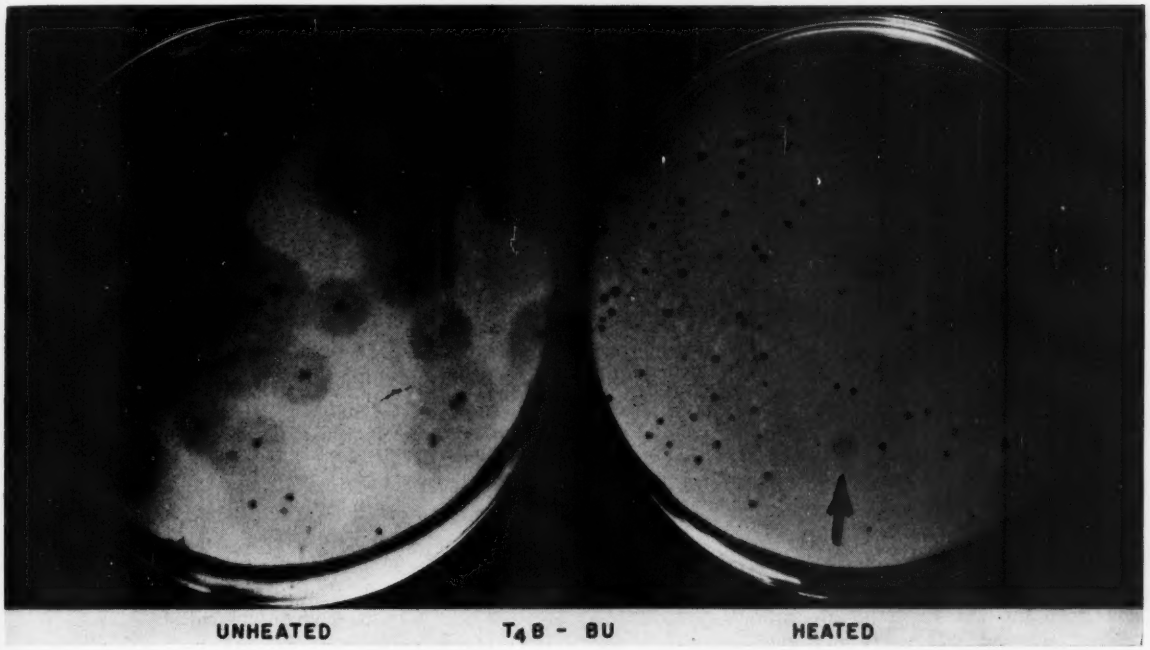

FIG. 7. Method for the detection of bacteriophage mutants producing lysozymes with heat stability properties that differ from that of the standard strain of phage. *Left:* T4B bacteriophage grown on an agar plate containing a suspension of *E. coli* B. The clear plaques result from successive rounds of infection and lysis of the bacteria. Lysozyme, produced in excess during the formation of phage, has diffused outward from each plaque. The *E. coli* cells, which are not ordinarily susceptible to lysis by lysozyme, become so after exposure of the plate to $CHCl_3$ vapors and one observes a "halo" of clearing around each plaque (15–17). *Right:* a duplicate plate which has been heated for a short time at 43°C before exposure to $CHCl_3$. Standard phage lysozyme is inactivated under these conditions. However a phage mutant that produces a more heat-stable enzyme should be detected by the retention of halo formation after heating. (The particular mutant indicated by the arrow has not been studied in detail. However the example illustrates the technique that has been used routinely in the genetic studies of Streisinger and Dreyer, (16)).

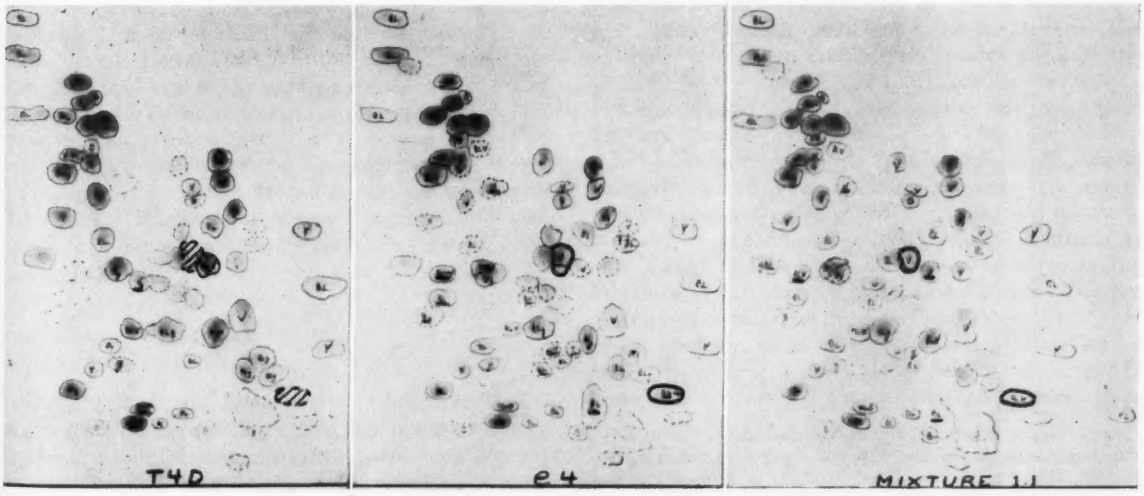

FIG. 8. Peptide maps of trypsin digests of lysozymes from the standard strain of phage, T4D, and from a mutant, e4, producing a more heat-labile enzyme.

structure following a point mutation, it is clear that no conclusions of biological significance can be made without the further study of a series of mutant enzymes. It will be of great interest to see how such data will support the present hypotheses derived from purely genetic considerations.

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Virus-induced acquisition of metabolic function

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THE DETAILED chemical study of the course of bacterial-virus multiplication has been in progress slightly less than 15 years, antedating the chemical study of the multiplication of animal and plant viruses by about a decade. This pioneering role for the bacteriophage systems stems from their exceptionally favorable attributes in such matters as ease of cultivation and assay of host and virus, the establishment of the time and multiplicity of infection, and so forth. Of some historical interest is the fact that the bacterial viruses subjected to the most detailed chemical exploration, i.e., the T-even phages T2, T4, and T6, are biochemical freaks. The unique properties of these phages have contributed to their extreme virulence, which, in itself, has facilitated the study of these viruses.

Initially we posed our problems in the somewhat trivial terms of the molecular mechanism of the extremely virulent activity of the T-even phages on *E. coli*. Many other laboratories have developed their problems in the more general terms of the molecular bases of inheritance for these same phages. In the past three years, the problems of virulence in these systems have been more or less solved in terms of the discovery of a series of rapid syntheses of many enzymes and proteins controlled by the insertion of viral DNA into the infected bacterium. Thus, the problems of the virulence of these phages can now be posed in the terms and problems of molecular genetics. Our progress in this area has now catapulted us into the same pot of molecular biology in which almost everyone else is stewing. In this paper, we summarize the unifying studies, of the past few years, of virus-controlled syntheses of enzymes and some other proteins in infected *E. coli*.

EARLY DATA ON POLYMER SYNTHESIS IN INFECTED CELLS

Until 1952, it was thought that the T-even viruses might merely redirect the existing functional metabolic machinery of the bacterium to the production of virus by replacing a set of critical bacterial templates by viral templates. Thus infected cells could no longer divide nor could they be induced to produce β-galactosidase (1). Nevertheless, respiration and assimilation into polymeric substance appeared to continue at the same rate after infection as before infection (2). If one concentrated on the fate of assimilated phosphorus, for example, this now was directed almost entirely toward the production of viral DNA, while the net synthesis of RNA—the nucleic acid present in largest amount in the bacterium—stopped. The discovery of the new and unique pyrimidine, 5-hydroxymethyl cytosine (HMC) (3, 4), for the first time led to the suspicion that the virus might be contributing another, qualitatively new element, i.e., the ability to control a metabolic function which, perhaps, did not exist at all in the uninfected bacterium.[2] This function was one essential to viral duplication in providing a new base, a pyrimidine without which viral DNA could not be made.

Almost from the beginning of chemical study, it had been appreciated that there was a mystery in protein synthesis in infected cells. As shown in Fig. 1, protein synthesis continued from the inception of infection (8); however, very little of the protein which was formed before viral DNA synthesis began, appeared in the virus which was eventually liberated (9, 10). Was this bacterial protein which continued to be synthesized? For example, it is known that infection causes a leak in the permeability barrier of the cell, and that there is a subsequent repair (11). It was possible that this repair involved protein synthesis.

It was demonstrated—first, by means of the specific analogue, 5-methyl tryptophan (12, 13), and, subsequently, with chloramphenicol (14, 15), that the early synthesis of protein was essential to the synthesis of viral DNA. Indeed, once this protein was made, the inhibition of further protein synthesis did not block the production of functionally active viral DNA (13, 15). It could, therefore, be asked what function was fulfilled by this early protein, which was not itself a component of virus, but was nevertheless essential to the production

[1] The work performed in the author's laboratory has been aided greatly by grants from the Commonwealth Fund, New York, N. Y. and the Upjohn Company, Kalamazoo, Mich.

[2] Although it had been known that deoxyribonuclease is markedly increased in amount after infection with certain viruses, and that this increase is inhibited by chloramphenicol and thienylalanine (5, 6), this effect was most frequently attributed to the activation of pre-existing enzyme, rather than to a *de novo* synthesis of the enzyme (7, 6). This preference probably reflected, in large part, the intellectual climate (at the time) relative to these problems, although evidence for both possibilities had been obtained. Whether one or the other, or both, is correct has not yet been established.

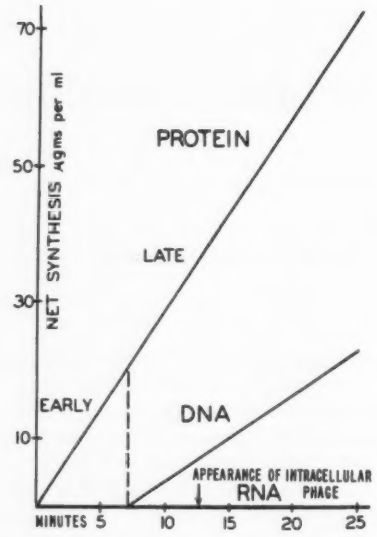


FIG. 1. Time course of polymer syntheses in *E. coli* infected by T-even phages.

of viral DNA. One obvious suggestion was that the early protein was involved in the formation of HMC. Having conceded the possibility of the expansion of the metabolic machinery, it would also be suggested that other enzymes might be made, particularly those which might account for the enormous stimulation of DNA synthesis observed on infection.

In Fig. 1, it can be seen that there is no net synthesis of RNA. The use of P^{32} then showed an incorporation of this isotope into the RNA fraction which was only 2% that of the incorporation into DNA (8). This result has been confirmed many times, but, although it was initially concluded that this amount of isotope in the RNA fraction reflected a contamination of the RNA, it has been shown rigorously that this P^{32} is, indeed, in RNA (16). This newly synthesized material is a chemically unique type of RNA, whose base composition mimics that of viral DNA. In addition, this RNA has an active turnover, in which the ribose nucleotides are converted to deoxyribonucleotides of viral DNA (16). It has been difficult to demonstrate a function for this RNA, but it has now been shown that the early steps of phage multiplication, which exclude DNA synthesis, require pyrimidines and adenine, presumably for RNA (17, 18).

It can be seen in Fig. 1, that DNA synthesis in infected cells stops for some minutes. DNA synthesis resumes at a markedly stimulated rate which, in some media (e.g., lactate), appears to compensate for the RNA that no longer accumulates (8). The new DNA, made after infection, is entirely viral, containing HMC and lacking cytosine (19, 20).

SIGNIFICANCE OF HYDROXYMETHYL CYTOSINE

The existence of the new base in viral DNA and the lack of cytosine, found normally in bacterial RNA and DNA, suggested that hydroxymethylation of cytosine to HMC converted the former base to a form unsuitable for normal bacterial syntheses. As we shall see, deoxycytidine triphosphate (dCTP), which is essential to the synthesis of bacterial DNA is, indeed, converted very efficiently in infected cells to deoxycytidylate (dCMP), the precursor of the HMC deoxyribonucleotide, hydroxymethyl deoxycytidylate (dHMP). Virulence and parasitism in this system are, thus, exhibited at the molecular level by appropriating a normal essential nucleotide for the formation of a unique viral metabolite. It should be noted that the host DNA is degraded in infection, and the dCMP therein contained is freed and converted to viral pyrimidine (21, 22).

In studies of the isolation of HMC derivatives from viral DNA, it was soon observed that nucleotides containing this base were present in a structure which resisted enzymatic release (23). Several workers (24–26) then showed that, in viral DNA, HMC was glucosylated at the hydroxymethyl group. It appears that the presence of glucose does, indeed, protect—to a considerable extent—phosphodiester bonds involving dHMP. Thus, viral DNA carries a significant molecular mechanism for self-protection when present in the DNase-rich medium of the infected cell. However this may not be the sole reason for the survival of viral DNA.

In addition, we can note that, although the base composition of the HMC viruses—T_2, T_4, and T6—are essentially identical (4), the glucose contents of these viruses differ considerably (26). Thus, T6 contains mainly large amounts of diglucosyl derivatives of HMC plus nonglucosylated HMC, T_4 contains only the monoglucosyl HMC, while T_2 contains mainly monoglucosylated and nonglucosylated HMC. HMC has thereby been implicated in mechanisms of parasitism and virulence, survival, and speciation among the T-even viruses.

BIOSYNTHESIS OF THYMINE AND HYDROXYMETHYL CYTOSINE

In exploring the biosynthesis of viral pyrimidine, it was found that exogenous orotic acid and pyrimidine of host DNA could be converted into viral HMC and thymine. The $—CH_2OH$ and $—CH_3$ of the latter bases were derived from the β-carbon of serine (27). The methyl group of thymine did not come from the methyl of methionine (28). A series of nutritional experiments with bacterial mutants and competition experiments with infected cells then revealed that the addition of the one-carbon fragments to form viral bases did not occur at the level of free bases or nucleoside (29). In order to test the syntheses at the nucleotide level, it was necessary to study these possible reactions in cell-free extracts.

Friedkin and Kornberg (30) demonstrated the presence of an enzyme, thymidylate synthetase, in extracts of normal *E. coli* which produced thymidylate (dTMP) from deoxyuridylate (dUMP), formaldehyde (HCHO), and tetrahydrofolic acid (THFA) (Fig. 2). Testing for

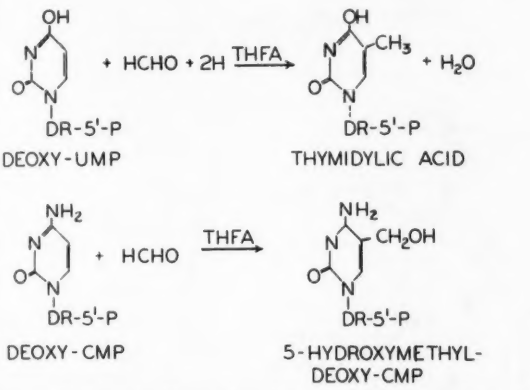


FIG. 2. Utilization of formaldehyde in the enzymatic synthesis of thymidylate and 5-hydroxymethyl deoxycytidylate.

similar reactions with dCMP in normal and T2-infected *E. coli*, Flaks and I showed that, with dCMP as a substrate, only extracts from infected cells were capable of fixing C^{14}-labeled formaldehyde in an acid stable form to yield dHMP, as in Fig. 2 (31, 32).

Although thymidylate synthetase was, indeed, present in uninfected *E. coli* strain B, the amount was shown to be markedly increased in phage-infected bacteria (31, 32). The mechanism of the reaction forming thymidylate does not appear to involve a free hydroxymethyl derivative, unlike the reaction which produces dHMP (30, 33). However, it was observed that hydroxymethyl deoxyuridylate and thymidylate were generated to a small extent during the conversion of dCMP to dHMP (33). This has been shown to be due to the appearance of a third enzyme, dCMP deaminase, in infected cells (Flaks, unpublished results) which not only deaminated dCMP but also deaminated dHMP. The appearance of this enzyme in viral infection has also been described recently in another laboratory (34).

PROPERTIES OF dCMP HYDROXYMETHYLASE

Three assays have been developed for the enzyme based on the fixation of C^{14}-labeled HCHO to dCMP to form radioactive dHMP (35). The ion-exchange method, reported in 1959 by Somerville et al. (36), is the most simple and accurate of the three, and is the method of choice, at present, in following the purification and other properties of the enzyme. The hydroxymethylase is completely inactive on cytosine ribonucleotide; however, cytosine arabinonucleotide appears to possess of the order of 0.6% of the substrate activity of dCMP (37). Using a purified enzyme preparation, the following Km values have been determined: 6×10^{-4} M for dCMP, 1.5×10^{-3} M for formaldehyde, and 1×10^{-4} M for THFA.

The separation of the hydroxymethylase and the thymidylate synthetase may be effected easily; the first enzyme is far more stable (33). A much greater degree of purification of the hydroxymethylase was accomplished on a diethylaminoethyl (DEAE) cellulose column, as described by Kornberg et al. (38) and, recently, in somewhat greater detail by Pizer and myself (35). Fig. 3 presents the elution from the column of the protein and enzyme derived from infected cells in 40 litters of Biogen culture containing 10^9 infected bacteria per milliliter. Phage multiplication was stopped, at 15 min, by addition of chloramphenicol to the culture, which was expelled onto ice; the cells were collected by centrifugation. The infected cells (40–50 g, wet wt) were frozen and thawed, and extracted twice in the Waring Blendor, for 1 min, in 250 ml of 0.05 M glycylglycine buffer, at pH 7.2 (0.001 M with respect to the glutathione). The extract, containing about 4 g protein, was centrifuged (at 30,000 rpm) for 30 min and, after removal of nucleic acids by precipitation with streptomycin, the fractionation of DEAE cellulose was effected by using two linear gradient elutions with phosphate buffer.

As can be seen in Fig. 3, a peak of enzyme activity is obtained at a low point in the protein elution, and the central tubes of this peak have a constant specific activity. The values presented in the graph were obtained for the purified fractions in this peak by reanalysis about 16 days after infection, and about 9 days after separation on the column. Specific activities of 2,200 were obtained by the column assay for key tubes 8 days before the time for the assays indicated on the chart. Extrapolation to the earliest point in the fractionation suggests specific activities, for the enzyme, of the order of 5,000—a value slightly lower than that obtained by Kornberg et al. A personal communication, i.e., 6,000—using our original, less reliable assay (32).

The peak tubes were pooled, concentrated, and examined by analytical ultracentrifuge and electrophoresis in 0.02 M KPO_4 buffer, pH 7.4, 0.1 M with respect to KCl. Two components of 85% and 15% were observed in both analyses. The major component had an $S_{20,w}$ of 4.4 and a mobility of -5.4×10^{-5} cm^2 volts^{-1} sec^{-1}. The minor component had an $S_{20,w}$ of 25 and a mobility of -9.3×10^{-5} cm^2 volts^{-1} sec^{-1}. Analysis of the specific activities in the peak of enzyme elution indicates that the enzyme would probably be a major protein component. From the $S_{20,w}$ for this component, assuming a frictional ratio of 1, a molecular weight of 49,000, and a turnover number of the order of 125 moles dCMP per mole of enzyme per minute have been calculated.

With these numbers, we may estimate the number of enzyme molecules per cell. In the Biogen experiment, 10^{10} infected cells gave rise to about 1 mg of extract protein capable of converting 0.3 μmole dCMP in 20 min. It can be calculated that an infected cell contains about 8,300 molecules of hydroxymethylase in 15 min, or produces about 9 molecules of enzyme per second over a 15-min interval. Since the appearance of enzyme occurs at almost a constant rate, it can be calculated that—at the time DNA synthesis begins (about 7 min)—the hydroxymethylase content of the cells can maintain

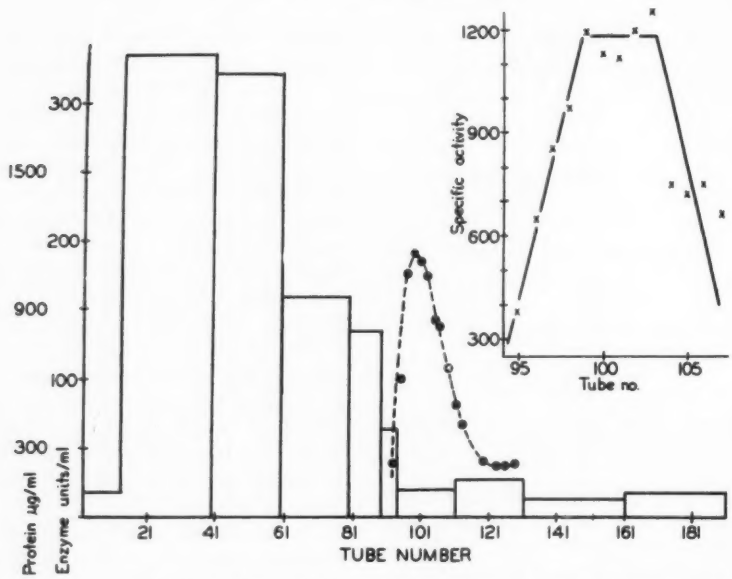


FIG. 3. Isolation of the deoxycytidylate hydroxmethylase by elution from a DEAE column. Dotted line = enzyme.

a rate of dHMP synthesis sufficient to produce 120 T2-virus particles per cell, if continued for 14 min. This value is close to the usual yield of a T-even phage per cell in a one-step growth experiment in the media used.

Pizer and I have now asked if any active enzyme is to be found in uninfected cells. To test this it has been necessary to run assays with approximately 2×10^7 cpm per assay tube or 2.4 µmoles C^{14} H_2O at 7 µc per micromole. Extracts of uninfected cells were prepared, and amounts of protein (1.6–4.7 mg) were used derived from 8.2×10^9 to 2.6×10^{10} cells. Among the blanks were used the reaction mixture minus protein, and the reaction mixture with extract lacking added dCMP. In another control, the reaction mixture was run with an extract of infected cells. The expected activity of enzyme was obtained despite the unusual radioactivity of the HCHO used. After 20 min, 0.55 µmoles DHMP was added as carrier, and this substance was purified initially on Dowex 50-H^+ and subsequently by paper chromatography in isobutyrate-NH_4OH. The nucleotide was hydrolyzed, and the free HMC was purified by paper chromatography in butanol-NH_4OH and isopropanol-HCl. The purification steps for carrier HMC are summarized in table 1. It can be seen that after the final chromatography, the activity present in carrier HMC in all assays was far less than could be expected on the hypothesis that the cells contained at least 1 molecule of dCMP hydroxymethylase per cell. Furthermore, there was no suggestion of a trend in fixation as the amount of cell protein was increased. It may be remarked that the exclusion of activity for 1 molecule of enzyme in any auxotroph or otherwise "deficient" bacterium does not seem to have been reported previously.[2a]

[2a] Note added in proof: The following experiments have been performed with Dr. Pizer since the submission of the manuscript. Cells were grown in the presence of S^{32} and infected in the presence of S^{35}. Fifteen minutes after infection cells were harvested and fractionated, as in Fig. 3, with numerous estimations of protein, radioactivity, and enzyme activity. The fractions containing enzyme possessed the highest radioactivity per milligram of protein. However, methionine isolated from this enzyme-containing fraction possessed a specific radioactivity of one-third that of the S^{35} in the medium. In addition to the possibility implied by this result that there is a protein or polypeptide precursor to the dCMP hydroxymethylase, it appears more likely that our enzyme preparation was not pure and was contaminated with unlabeled host protein, since the enzyme preparation reacted strongly with an antiserum prepared against protein isolated from normal cells by the method used in isolating the hydroxymethylase.

As a result of the observations of heterogeneity in the enzyme preparation, we have undertaken sedimentation studies of enzymatic activity in a partition cell and diffusion measurements by a porous disc method. The analyses have revealed an $S_{20,w}$ of 4.0 S and $D_{20,w}$ of 6.1×10^{-7} cm²/sec. The molecular weight of the enzyme has been calculated to be 64,000 ± 9,000. It is obvious that if the specific enzyme activity can be increased by a factor of 3, and the molecular weight is only slightly changed, the turnover member will approximately triple, and the total number of enzyme molecules per cell and the rate of enzyme synthesis will fall to about one-third that indicated in the text. On the other hand, the calculation of turnover number from an observed specific activity of 1,200 and a molecular weight of 64,000 leads to a turnover number of about one-third that used in Table 1. In either extreme the presence of active dCMP hydroxymethylase in uninfected cells appears to be excluded by the data in Table 1.

BIOSYNTHESIS OF dCMP HYDROXYMETHYLASE

Enzyme cannot be detected in uninfected cells, but is found in large amount in all infected cells (8,300 molecules/cell, or 0.7% of the protein extracted at 15 min, since each infected cell can give rise to virus containing HMC. Several possibilities can be visualized concerning the appearance of the enzymatic activity.

1) The virus injects the enzyme. In addition to the

TABLE 1. *Exclusion of Active dCMP Hydroxymethylase in E. coli*

Assay Tube	Chromatography			Expected cpm for 1 Molecule Enzyme/Cell†
	Nucleotide	Free base		
	Isobutyrate-NH_4OH	Butanol-NH_4OH	Isopropanol-HCl	
*mg protein**	*cpm per 0.55 μmole*			
0	9900	890	22	0
1.56	5050	440	15.4	251
3.12	4450	288	3.7	502
4.68	2800	332	6.4	753
3.12 + 0 dCMP	1430	120	0.5	0

* 1 mg protein = 5.6×10^9 cells. One molecule of enzyme (TN 125)/cell would produce 2.3×10^{-5} μmoles dHMP per mg protein per 20 min. Formaldehyde-C^{14} was used at 7×10^6 cpm per μmole.

† See footnote 2a.

fact that we have been unable to detect enzyme in disrupted virus preparations (39), it can be calculated that the weight of the enzyme found in infected cells (7×10^{-16} g) approximates the entire weight of a T-even phage ($5 - 7.5 \times 10^{-16}$ g). This hypothesis does not appear reasonable.

2) The enzyme is present in uninfected bacteria in an inhibited state. We have disrupted bacteria in a variety of ways without revealing enzymatic activity. In addition, we have mixed and incubated extracts of infected and uninfected cells without inhibiting enzymatic activity or producing an increase in this activity (39). This shows the absence of excess inhibitor in uninfected cells and the absence of a system in extracts of infected cells capable of activating the hypothetical, inhibited complex in uninfected cells.

3) The enzyme is synthesized after insertion of the phage contents into the bacterium. The conditions required for the appearance of the dCMP hydroxymethylase bear on this hypothesis.

a. Infection must be effected with a T-even phage; enzyme is not formed during infection with other T phages, such as T1 or T5, or after induction of a lysogenic system such as K12-λ (39). Thus, infection with HMC phage is required for the development of the activity.

b. Osmotically shocked ghosts of T-even phages, although capable of adsorbing to the bacteria and affecting bacterial metabolism, cannot induce enzyme. The contents of the phage head are therefore essential for the appearance of enzyme. These contents consist mainly of DNA and small amounts of a specific internal protein (40), a polypeptide (41), and polyamines (42). The polyamines may be replaced by other cations in the phage without affecting phage viability (43).

c. The contents of the phage head do not contain free pyrimidine deoxyribonucleotides (39), which might be imagined to induce the biosynthesis of the hydroxymethylase.

d. As presented in Fig. 4, ultraviolet irradiated phages induce the appearance of the enzyme in normal amount under conditions (15 hits, virus:cell = 4) in which virus cannot multiply[3] or induce the formation of DNA (39). This work has been extended to a study of single infection with irradiated phage using the column assay. It appears that the site, in the phage, which controls the appearance of enzyme has a sensitivity to ultraviolet light about one-twentieth that of all the sites which control virus multiplication. (N. Delihas, in a personal communication). Assuming that the target for ultraviolet inactivation is phage DNA, all the major portions of which are equally sensitive to radiation, one may suggest that the portion of DNA controlling hydroxymethylase synthesis is a small fraction (perhaps about 1/20) of the total DNA but, nevertheless, this site is quite large (about 10,000 nucleotide pairs, or a molecular weight of about 6×10^6).

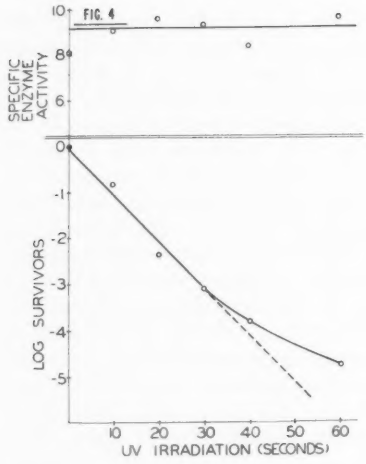
It is essential, therefore, to insert a large viral polynucleotide into the bacterium. Once the DNA has entered the cell, enzyme appears at an almost constant rate for 10–15 min. We have shown that protein synthesis is essential for enzyme production (39). Thus, if protein synthesis is blocked by 5-methyl tryptophan, as in Fig. 5, or by deprivation of an amino acid requiring mutant, enzyme production does not occur. Enzyme synthesis can be begun by a later supply of the appropriate amino acid to the inhibited or deprived cell. Thus, the prerequisite of protein synthesis prior to synthesis of viral DNA is indeed explained, in part, by the requirement of protein synthesis for the appearance of the dCMP hydroxymethylase. Nevertheless, it can still be supposed that the protein which is synthesized is not the enzyme itself but, rather, a protein necessary to free the pre-existing inhibited enzyme or otherwise to activate an inactive or incomplete enzyme. Although we do not think that this is likely, we are currently attempting to disprove this by demonstrating a *de novo* synthesis of the enzyme from metabolites supplied exogenously after infection.

BIOSYNTHESIS OF THYMIDYLATE SYNTHETASE

Our studies with this enzyme originated, in part, as a result of the similarities of the early methods of assay for synthetase and hydroxymethylase (31). From the fixation of acid-stable $C^{14}H_2O$, it was shown that *1)* this enzyme was normally present in *E. coli* capable of synthesizing thymine; *2)* the enzyme rapidly increased sixfold to sevenfold during infection of strain B with T2; *3)* the increase did not occur on infection with ghosts; and *4)* the increase of enzyme required protein synthesis (33).

Extending this work, Barner and I explored thymine-requiring mutants, which had been observed to syn-

[3] *E. coli* strain W will adsorb T2 and be killed by this phage, without giving rise to DNA and virus (23). Despite the abortive quality of the infection, this strain will produce dCMP hydroxymethylase (Pizer and Cohen, unpublished results).



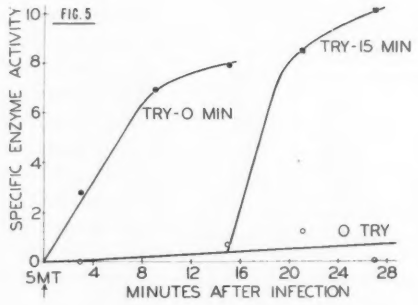


FIG. 4. Effect of ultraviolet irradiation of virus on hydroxymethylase formation. Cells were multiply infected with virus irradiated to varying levels of survival. The enzyme contents of such infected cells were determined.

FIG. 5. Effect of 5-methyl tryptophan on formation of hydroxymethylase. 5 MT = 5 methyl tryptophan. Try = tryptophan. Cells were infected in the presence of 5 MT and tryptophan was added in two systems at different times and omitted in a third system. The development of the hydroxymethylase was determined.

thesize thymine after infection with T2 (44) and T5 (45). Using a sensitive carrier assay, it was shown that although the extracts of thymine-requiring strains lacked the synthetase, infection with T2 or T5 resulted in the extensive production of the synthetase (46). It was found that in infections with T5, a virus which does not contain HMC, production of the synthetase occurs without a concomitant production of the dCMP hydroxymethylase. Indeed, production of the synthetase in T5-infected cells exceeds that in T2-infected cells.

We have obtained two highly active inhibitors for this enzyme, which is crucial in the economy of most cells. In the absence of thymidylate synthesis and exogenous thymine, a normal synthesis of DNA cannot occur. The unbalanced cell growth, which then ensues, results in the irreversible loss of the ability to multiply or "thymineless death" (44). Such a situation can be provoked by 5-fluorouracil deoxyriboside in many cells, including tumors, since the nucleotide, fluorodeoxyuridine-5′-phosphate, is a potent inhibitor for thymidylate synthetase (47). Fluorouridine-5′-phosphate is essentially inactive.

In the search for a fluorouracil nucleoside which is less readily degraded by the mammal, Dr. J. Fox of the Sloan-Kettering Institute has synthesized D-arabinofuranosyl 5-fluorouracil, and we have prepared the 5′-phosphate of this nucleoside by an enzymatic method. This new nucleotide (F-aUMP) is also an inhibitor of thymidylate synthetase, although one-hundredth as potent as F-dUMP. In the study of compounds which can provoke thymineless death by this mechanism, T2 or T5 virus-infected bacteria are easily the best source of the thymidylate synthetase.

The production of thymidylate synthetase can be controlled by three chemically different DNA's. These are the cytosine-containing DNA of strain B, the HMC-containing DNA of T-even phages used to infect strain B_T^-, and the cytosine-containing DNA of T5.[4] It was, therefore, of interest to see if the three forms of the enzyme were different, and the two inhibitors, F-dUMP and F-aUMP, were used to compare the sensitivities of the active sites of the enzyme in extracts of strain B and of virus-infected B_T^-. Miss H. D. Barner has recently improved the assay greatly for these experiments. As can be seen in Fig. 6, the active sites of the bacterial enzyme and two viral-induced types of thymidylate synthetase are indistinguishable by this test.

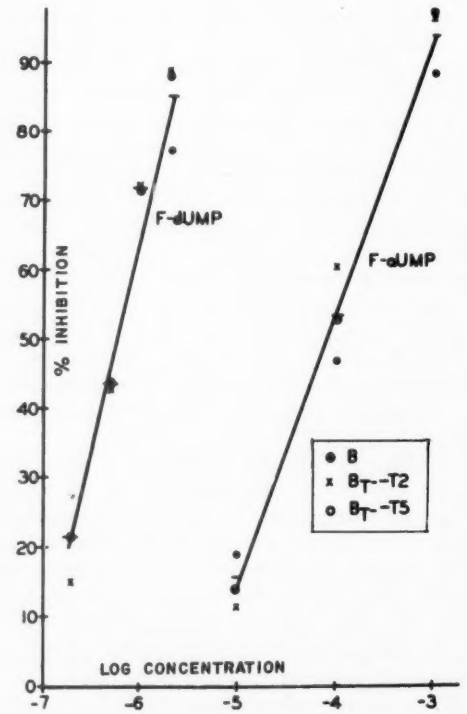


FIG. 6. Inhibition of two fluorouracil nucleotides on thymidylate synthetases induced by chemically different DNAs. F-dUMP = Fluorodeoxyuridine-5′-phosphate. F-aUMP = D-arabinofuranosyl fluorouracil-5′-phosphate.

[4] Of course, we cannot yet state that there are chemical differences between the fractions of *E. coli* DNA and T5 DNA which control the production of this enzyme, but the over-all compositions of the total DNA's of these forms are quite different (4).

OTHER VIRUS-INDUCED ENZYME AND PROTEIN SYNTHESES

In Fig. 7 is presented a summary of new and stimulated syntheses induced in infection by a T-even phage. After the initial description of the appearance of the three systems presented earlier—dCMP hydroxymethylase, thymidylate synthetase, and dCMP deaminase—a number of other laboratories undertook to study a cellular system in which the insertion of a chemically unique viral DNA induced the rapid production, in all cells, of many unique enzymes essential to the rapid multiplication of the viral DNA and many other viral-specific proteins. Many questions could still be posed concerning the exclusion of cytosine, and the steps involving purine nucleotides, DNA polymerase, glucosylation, and so forth, in the synthesis of virus DNA.

In testing to see whether the DNA polymerase of T2-infected cells was specific for HMC nucleotides, it was observed that infected cells elaborated a specific dCTPase, which removed pyrophosphate from dCTP to form dCMP (36, 38, 48). Thus, a compound essential for the formation of bacterial DNA was eliminated, i.e., dCTP, providing the dCMP essential for the formation of dHMP via the hydroxymethylase. The dCTPase is not produced in T5 infection.

According to Koerner (in a personal communication), the activity of dCTPase in T2-infected cells is ten times as much as is necessary to generate the dCMP as a substrate for hydroxymethylation. It may be pointed out, as well, that this is also consistent with the possibility that all deoxyribose is derivable via cytosine nucleotides.

Several laboratories (36, 38) have also described the phosphorylation of dHMP and the production of dHDP and dHTP. Although I have presented this as two new steps, in this reaction and, indeed, in the activation of all the deoxyribonucleotides to the triphosphate level, it is not certain that two separate, specific enzymes are involved. The dCMP kinase of *Azotobacter vinelandii* forms only the diphosphate (49), and the conversion of dCDP to dCTP can be effected to dCTP by phosphoenol pyruvate and pyruvic kinase. It would be of interest to know whether pyruvic kinase fulfills this role in infected cells for other deoxynucleoside diphosphates. No dCMP kinase is produced in *E. coli* after T2, T3, and T7 infection; however, a marked increase in activity appears in T5 infection (38, 50).

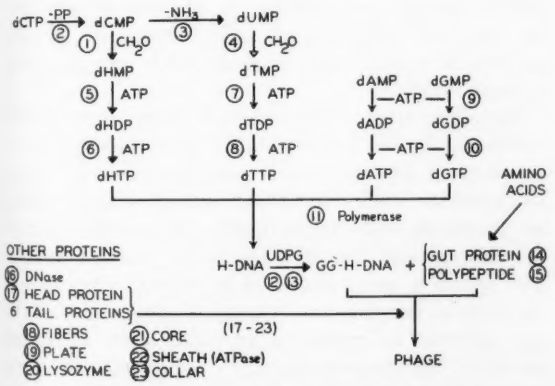


FIG. 7. New and stimulated reactions in T6. Circled numbers = new or stimulated units. Filled in arrows = new proteins. Hollow arrows = either stimulated or new enzyme.

Tenfold to 20-fold increased activities have also been observed in T2, T4, T6, and T5 infection for dGMP kinase and dTMP kinase, but not for dAMP kinase. The last appears to be present in uninfected cells in excess (38, 50). The increase in thymidylate kinase is blocked by chloramphenicol, suggesting the synthesis of protein (50). As in the case of the thymidylate synthetase, described earlier, it may be asked if the increase in these kinases represents more of the old enzymes or of new types of polypeptides having similar active sites. Bessman and van Bibber (51) have reported that the dGMP kinase formed after infection no longer has the requirement for K^+ that is characteristic of the kinase formed before infection.

Given the dHTP and the other normal triphosphates, several groups of investigators (38, 48) have studied the polymerase present after infection and its activity in the synthesis of a DNA containing HMC. About a tenfold increment has been seen in the DNA polymerase, and the purified enzyme of both uninfected and infected cells appears to be able to handle dCTP as well as dHTP. In these tests heated DNA derived from T2 or calf's thymus can be used interchangeably.

Koerner et al. (48) have prepared a monoglucosyl dHTP, and have observed that this does not participate in the reaction with DNA polymerase. On the other hand, according to Kornberg and his collaborators (38), DNA containing HMC, generated from dHTP and DNA polymerase, can be monoglucosylated in part by uridine diphosphate glucose (UDPG) in the presence of a new enzyme formed in bacteria infected by T2. Extending this important observation (A. Kornberg, in a personal communication), this group has found that two separable enzymes are formed in T4-infected cells, one which adds on a small amount of glucose to T2 DNA, and a second which converts T2 DNA to a form in which all HMC is completely monoglucosylated. Two separable enzymes are also present in T6-infected cells, one like that in T2-infected cells and a second that does not monoglucosylate, but adds a second glucose to T2 DNA and T4 DNA.

With the apparent synthesis of 13 enzymes in the first minutes after infection, we are finally in a position to account, in outline at least, for the stimulated DNA synthesis first observed so many years ago (8). These phage-infected bacteria are evidently a source par excellence for the study not only of the individual enzymes, since they are present in extraordinarily high concentration, but also of the specific control and kinetics of protein synthesis by specific DNA. However, in addition there are many other problems which remain in this system, e.g., when are the other

viral proteins produced, and how are these syntheses related to the production of viral DNA?

Of the viral proteins, the internal "gut" protein of Levine et al. (40) is unique in appearing with the enzymes of DNA synthesis. With an immunochemical method, this specific protein is detectable 2–3 min after infection (52). As with the hydroxymethylase, infection with ultraviolet-irradiated phage induces its formation despite the prevention of DNA synthesis. This synthesis of internal protein is not blocked by proflavine, but is prevented by chloramphenicol. It appears to be significant that if this inhibitor is added at various times early in infection, a correlation appears to exist between DNA-synthesized and internal protein formed prior to addition of the agent. When the inhibitor was added 10 min after infection, the rate of DNA synthesis remained high, until the phage equivalents of DNA approached that of internal protein. At this point, the rate of DNA synthesis fell (52).

According to Hershey (41), the formation of the polypeptide, but not the polyamines, is also blocked by chloramphenicol. The recent studies of Kellenberger et al. (53), showing the late formation of a chloramphenicol-sensitive principle essential to the condensation of elements of the DNA pool, raises the problem of the possible relation of this polypeptide to the condensing principle and its mechanism of action.

Following the condensation of phage DNA, between 9 and 11 min after infection, it appears that centers are produced on which may be constructed the numerous structures (at least seven) of the phage head and tail. Of these, at least two possess enzymatic activity essential for the penetration of phage DNA into the bacteria. These are lysozyme (54) and an ATPase (55). It has not been reported when these enzymes are elaborated or whether or not the appearance of the activity and the phage structure take place simultaneously.

CONCLUDING REMARKS

The phenomena of the appearance of new enzymes, described above will certainly not be confined to the T phages. The development of a polysaccharidase in Klebsiella infected by a phage was described some years ago by Adams (56) and by Park (57). In this interesting case, the polysaccharidase, which hydrolyzed the bacterial capsule, was found both in a soluble form in the lysate and associated with the phage. The enzyme, thus, appears to facilitate attachment of the phage to the bacterium.

Several similar phenomena have been described in the animal-virus system. The appearance of neuraminidase in myxovirus-infected cells is well known, although inadequately explored. More recently, Rogers (58) has described the appearance of arginase in rabbit epithelium infected by rabbit papilloma virus. Thus, the acquisition or increase, or both, of metabolic function as a result of the addition of the viral genome to the infected cell will have the widest significance in explaining not only parasitic mechanisms, but the possible mode of action of tumor viruses as well. We have indicated the existence of 23 proteins whose production is controlled by the insertion of T-even phage DNA. Unquestionably a number of other enzymes will be found to be increased, e.g., the formation of deoxyribose, and so forth. On the other hand, other systems, such as respiration and amino acid activation, appear not to be increased, and these can be conceived as possibly rate-determining in the system as a whole.

Since bacterial DNA is degraded very soon after infection, it is reasonable to suppose that the code determining the amino acid sequence in each new protein is contained within the nucleotide sequence of viral DNA. Although HMC and its glucosylated derivatives can readily be conceived as equivalent to cytosine in this sequence, it is unlikely that all elements for this sequence controlling a single protein will be functionally identical for all amino acids; it is also quite reasonable to imagine that bacterial and virus-induced proteins will not be entirely identical in amino acid sequence. Whether this is true, remains to be determined. The solution of this question is only the first step pointing to one of the fundamental questions of molecular biology, i.e., the correlation of the nucleotide sequence in DNA with amino acid sequence in the protein whose structure is controlled by the DNA.

The problem of the intermediate steps in the control of protein synthesis by viral DNA is also one of the utmost importance. It can be inferred, although proof can scarcely be considered adequate, that the early RNA synthesis is functionally related to the synthesis of the new enzymes and other proteins. It is a general phenomenon, in these early protein syntheses, that they come to a halt at about 15 min after infection. Why do they stop? Is there a competition for amino acids between enzymes and viral coat proteins, or is there a destruction of the specific RNA templates by conversion of component ribonucleotides to the deoxyribonucleotides of viral DNA, or both? Is the turnover of RNA templates and competitive relation to protein and DNA a general phenomenon, which may account for the fact that differentiated cells elaborating specific proteins do not normally produce DNA and duplicate chromosomes?

Although, in a technical sense, these systems are admirable for the exploration of these questions, it is now evident that the T-even phages contain too much DNA and too much information, that too many proteins are produced, and that multiplication in cells infected by these phages requires too many parallel events. This system is too complicated to permit us to relate a twentieth of the genome in chemical terms to one of the new proteins. Of course, if we could insert a defined portion of the genome at will, we would be in a much better position. However, *E. coli* has never been transformed nor has the DNA of a T-even phage ever been reproducibly fractionated.

It may be suggested that the pursuit of these problems requires an infectable cell, transformable at high efficiency, and a tiny phage, possessing a bare minimum of DNA necessary for the inheritable transmission of virulence. Is the bare minimum of DNA that in $\phi \times 174$, and how many proteins does the DNA complement of this phage control? If only a single polypeptide chain is controlled in infections by $\phi \times 174$, we may cease to worry about the requirement for transformability. However, if this DNA determines the biosynthesis of several different polypeptides, we shall wish to fractionate the DNA and introduce the determinants separately (as in bacterial transformation). We are at the stage, therefore, at which the study of the molecular relations of DNA as a genetic determinant and the protein produced as the expression of this determinant will be markedly facilitated by finding new and more simple biological systems to replace our old friend, T2.

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Interaction of viral nucleic acids with mammalian cells[1]

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THE NOW CLASSICAL EXPERIMENTS of Hershey and Chase (1) provided the first demonstration of the unique importance of bacteriophage deoxyribonucleic acid in the reproduction and genetic continuity of these viruses. The corresponding central role played by the ribonucleic acid (RNA) of tobacco mosaic virus (TMV) was conclusively and directly demonstrated some four years ago by Gierer and Schramm (2). They showed that RNA, isolated from this virus, was capable of initiating an infection, in tobacco leaves, leading to the formation of virus particles identical with those from which the nucleic acid was isolated. The same report included a description of a new technique for the isolation of RNA—a technique which has come to be known simply as the phenol method.

During the past three years, this method has been used successfully for the isolation of infectious RNA from what is now a fairly imposing number of mammalian viruses. These viruses are listed in table 1. It might be noted, in passing, that they are all small and, for the most part, are agents with neurotropic potentialities.

The infectivity of these viral RNA preparations has been demonstrated in mice by intracerebral and intramuscular injection; in the embryonated egg, by inoculation into the allantoic cavity and onto the chorioallantoic membrane; and in vitro by inoculation of monolayers of cell types susceptible and insusceptible to the parent virus.

In general, the viral RNA preparations have exhibited a very low degree of infectivity compared to that of the virus suspensions from which they have been prepared. Their activity has been of the order of 0.1% that of the corresponding virus suspensions. One, and perhaps the major, reason for this apparent low activity will be clear from the studies to be described.

Infectious RNA is, perhaps, most readily tested in an in vitro system: extracellular ribonuclease can be removed by washing, the conditions under which cells and RNA interact can be carefully controlled, and the results can readily be measured. For quantitative studies of the cell-RNA interaction, a suspended, single-cell system is preferable to a monolayer, since, under these conditions, the statistics of discrete distributions can readily be applied, and the cell population uniformly exposed to modifications of the environment in a precisely quantitated fashion.

The results of studies of the system composed of Mengo encephalomyelitis virus RNA and Earle's "L" strain mouse fibroblast line of cultured cells comprise the subject of this presentation. This system is a convenient one, since Mengo virus has been found to lyse "L" cells and to produce plaques in "L"-cell monolayers, and because these cells grow well on glass or in suspended cell culture (13, 14).

THE MENGO RNA—"L" CELL SYSTEM

Infectious Mengo RNA. Mengo RNA was extracted from 10% (w/v) suspensions of infected Ehrlich ascites tumor cells by the phenol method. RNA was precipitated at 0 C from aqueous solution by the addition of solid NaCl to a 1-M concentration. The precipitate was collected by centrifugation and redissolved in one-quarter the original volume of 0.14-M NaCl in 0.02-M phosphate buffer, pH 7.3 (0.14-M PBS). Solutions prepared in this manner were designated 4×10^{-1} RNA. Aliquots could be stored at −70 C for several months without any significant decrease in biological activity.

Tissue Culture. Earle's "L" strain mouse fibroblasts were grown in suspended culture in Earle's saline (without calcium and with ten times the usual concentration of phosphate), containing double the concentration of vitamins and anino acids recommended by Eagle, and 10% horse serum. In what follows, this mixture will be referred to as 2 × Eagle's in Earle's medium.

For the preparation of monolayers, cells were collected by centrifugation of the spinner cultures, and resuspended to a concentration of 10^6 cells per milliter in 2 × Eagle's in Earle's medium. Aliquots of 6 ml of the suspension were pipetted into 60-mm petri dishes, which were then incubated for 2–3 hours in an atmosphere of 5% of CO_2 in air, to permit the cells to settle out, adhere to the glass, and form monolayers.

Titration of Infectious RNA. The infectivity of the Mengo

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[2] Traveling Fellow of the New South Wales State Cancer Council.

TABLE 1. *Mammalian Viruses From Which Infectious Ribonucleic Acid Has Been Isolated*

Virus	Reference
Mengo encephalomyelitis	Colter, Bird, and Brown (3)
West Nile	Colter, Bird, Moyer, and Brown (4)
Poliomyelitis	Colter, Bird, Moyer, and Brown (4)
Poliomyelitis	Alexander, Koch, Mountain, Sprunt, and Van Damme (5)
Eastern equine encephalitis	Wecker and Schäfer (6)
Western equine encephalitis	Wecker (7)
Semliki Forest	Cheng (8)
Encephalomyocarditis	Huppert and Sanders (9)
Foot-and-mouth disease	Brown, Sellars, and Stewart (10)
Murray Valley encephalitis	Ada and Anderson (11)
Echo (1 and 8)	Sprunt, Redman, and Alexander (12)
Coxsackie (A-7, B-4, B-5)	Sprunt, Redman, and Alexander (12)

RNA preparations was measured by incubation with "L" cells in suspension, followed by an examination of the treated cells for infectious centers. The details of this method, which has not previously been used for the assay of infectious RNA, are as follows.

Aliquots of a logarithmically growing suspended cell culture, containing the desired number of cells, were centrifuged in stoppered, 12-ml centrifuge tubes. The cells were washed once in Hanks' balanced salt solution and recentrifuged. Following a brief temperature equilibration at 37 C, the cells were suspended in a solution of RNA (usually 1.0 ml) in the diluent to be tested, and placed in a 37-C water bath. After 15 minutes' incubation, during which the tubes were agitated manually every few minutes to keep the cells uniformly suspended, the cell-RNA mixture was diluted to 10 ml with 2 × Eagle's in Earle's medium. The cells in this mixture were then titrated for infectious centers. With the higher concentrations of RNA, it was necessary to dilute further the mixtures before titration to reduce the plaque counts to readable numbers—that is, less than 100/plate.

Titration of Infectious Centers. Two 4.5-ml aliquots of each mixture were pipetted onto monolayers, which were incubated for 1 hr at 37 C, in an atmosphere of 5% CO_2 to permit the test cells to sediment and adhere to the indicator layer. The medium was then carefully removed, and the monolayers were covered with 4.5 ml of an agar overlay. The overlay consisted of 1% of agar in Hanks' balanced salt solution containing 0.1% of yeast extract, 0.17 % of bovine plasma albumin, 0.26% of $NaHCO_3$, 1:40,000 neutral red, double the usual concentration of Eagle's nutrients, and penicillin as well as streptomycin. Incubation at 37 C was continued. Plaques were counted on the second and third days.

Validity of System. The pertinent characteristics of this method of titrating infectious centers are listed below.

1) A linear relationship exists between the number of plaques formed and the dilution of the cell suspension. To test this, cells were incubated with a high concentration of RNA. Further dilutions of the treated cell-suspension were made, and aliquots of these dilutions assayed for infectious centers. Over the range 0–100 plaques per plate, there was a strict linear relationship between plaque count and dilution of the cells. There was some departure from linearity when counts were higher than 100 per plate, owing to confluence of plaques.

2) Dilution of the RNA-cell mixture with culture medium prevents any further cell-RNA interaction. When cells were suspended in RNA and simultaneously diluted with culture medium, no infectious centers were formed. Also, RNA, in the absence of cells, diluted with medium and pipetted onto monolayers, produced no plaques. That is, there was no carry-over of infectious RNA units to the monolayer, other than those which had become stabilized by interaction with cells.

3. Incubation for 1 hour is adequate for the attachment of infected cells to the indicator monolayer. When fluids were carefully removed after 1 hour and placed on fresh monolayers, no plaques were formed.

4. The total number of cells put on the monolayer does not affect the number of plaques formed from a standard number of infected cells. Addition of increasing numbers of uninfected cells, up to a maximum of 10^6 cells, produced no variation in the plaque counts greater than that expected on the basis of chance.

Effect of Osmolarity of Diluent on RNA-"L" Cell Interaction. It has been reported previously by others (15, 16). that when solutions of poliovirus RNA, applied to monolayers of susceptible cells, were made 0.8 M with respect to NaCl, there was a marked increase in the number of plaques formed, as compared with the number formed in 0.14-M PBS. This prompted an investigation of the effects of hypertonicity of the medium on RNA-cell interaction in the suspended cell system. Representative data are shown in table 2.

TABLE 2. *Relationship Between Solute Concentration and Establishment of Infectious Centers From Mengo Ribonucleic Acid Preparations*

Molarity of Sodium Chloride in RNA Solution*	Total Number of Infectious Centers†	Molarity of Sucrose in RNA Solution‡	Total Number of Infectious Centers
0.14	13	0.0	13
0.45	51	0.4	191
0.52	224	0.6	317
0.57	254	0.7	381
0.62	250	0.8	237
0.67	270	0.9	107
0.72	153	1.0	45
0.77	104	1.2	29
0.87	23		

* Solution also contained 0.02-M potassium phosphate buffer, at pH 7.3. † Developed by 10^6 washed "L" cells suspended in 1.0 ml of a 5×10^{-2} dilution of Mengo RNA in the indicated diluent, after 15 minutes at 37 C. ‡ Solution also contained 0.14-M sodium chloride and 0.02-M potassium phosphate buffer, at pH 7.3.

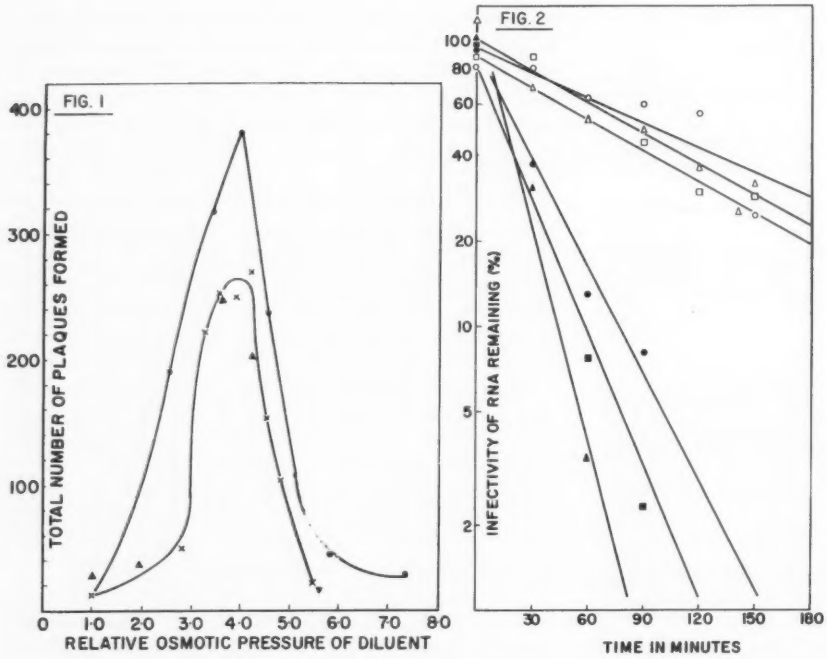

FIG. 1. Relationship between the formation of infectious centers in "L" cell—Mengo RNA mixtures and the osmotic pressure of the diluent. 10^6 cells incubated in 2.5×10^{-2} RNA for 15 minutes at 37°C. (●) Plaques produced after incubation of cells and RNA in sucrose-0.14 molar PBS media. (△, X) Plaques produced after incubation of cells and RNA in phosphate-buffered sodium chloride media.

FIG. 2. Thermal inactivation of Mengo RNA. Residual activity after incubation at 37°C was estimated by measuring ability to form infectious centers in "L" cells. ○, ●—in 0.14 molar sodium chloride-0.02 molar phosphate buffer, pH 7.3. □, ■—in 0.64 molar sodium chloride-0.02 molar phosphate buffer, pH 7.3. △, ▲—in 0.7 molar sucrose in 0.14 M PBS. Closed symbols = in absence of versene. Open symbols = in presence of 10^{-3} M versene.

As the molarity of the NaCl was increased from physiological, in 0.02-M phosphate buffer, there was a progressive increase in the number of infectious centers formed in a given volume of Mengo RNA, up to a maximum at 0.65-M NaCl. At molarities greater than 0.65, the number of infectious centers formed dropped sharply.

To test whether or not the hypertonic NaCl solutions could be producing these effects primarily by dehydrating the cells, the effects of sucrose on the cell-RNA interaction were examined. Cells and RNA were incubated in solutions of sucrose in a phosphate-buffered physiological saline solution. The results of one such experiment are shown in table 2. With increasing concentrations of sucrose in 0.14-M PBS, the number of infectious centers produced increased to a maximum at 0.7-M sucrose. At higher sucrose concentrations, the number of infectious centers produced dropped very sharply.

These data are presented somewhat differently in Fig. 1. Here the number of infectious centers produced in "L" cell-RNA mixtures is plotted against the relative osmotic pressures[3] of the diluents in which cells and RNA were incubated. It can be seen that, for both the NaCl and sucrose series, the maximal number of infectious centers is formed when the osmotic pressure of the medium in which cells and RNA interact is 4.0–4.1 times that of a physiological saline solution. The total number of plaque-forming units established in the optimal sucrose medium exceeds the number formed in the optimal sodium chloride solution by a factor which has ranged, in an extensive series of experiments, from 1.5 to 2.

Influence of Sodium Chloride and Sucrose on Mengo Virus-"L" Cell Interaction. It was of interest to see if the intact Mengo virus behaved differently from its naked RNA in solutions of high osmolarity. The system used was the same as that used for the RNA, except that—after incubation of the

TABLE 3. *Influence of Sodium Chloride and Sucrose Concentration On Mengo Virus-"L" Cell Interaction*

Diluent	Total No. of Infectious Centers*	Percentage Recovery
0.14-M PBS†	522	100.0
0.37-M PBS	0	<0.2
0.47-M PBS	0	<0.2
0.57-M PBS	0	<0.2
0.0-M Sucrose‡	522	100.0
0.3-M Sucrose	114	21.8
0.5-M Sucrose	75	14.4
0.7-M Sucrose	13	2.5

* Obtained from 10^6 washed cells suspended in 1 ml of the virus preparation and exposed for 15 min at 37 C. † XM PBS = x molar sodium chloride in 0.02-M potassium phosphate buffer, at pH 7.3. ‡ XM Sucrose = x molar sucrose in 0.14-M sodium chloride–0.02-M phosphate buffer, at pH 7.3.

[3] Calculated from the equation

$$\text{Osmotic pressure} = nm\phi\left(\frac{RTW_A}{1{,}000V_A}\right)$$

when n equals number of moles of ions formed from 1 mole of electrolyte, m equals moles of solute per kilogram of solvent (molal concentration), and ϕ equals the osmotic coefficient. The osmotic pressures of the saline and sucrose solutions were expressed as multiples of the osmotic pressure of 0.14-M PBS.

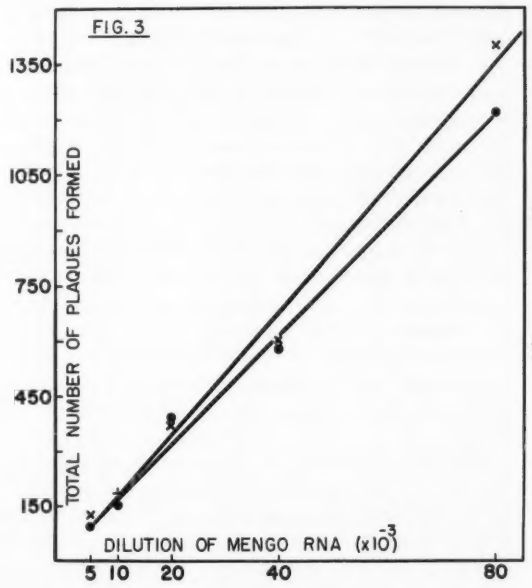

FIG. 3. Relationship between the number of infectious centers formed and *1*) concentration of RNA, *2*) volume of RNA solution. 10^6 cells incubated 15 minutes at 37° centigrade in (●) 1 milliliter of RNA solution, (X) 2 milliliters of RNA solution. Diluent = 0.7 molar sucrose in 0.14 molar PBS.

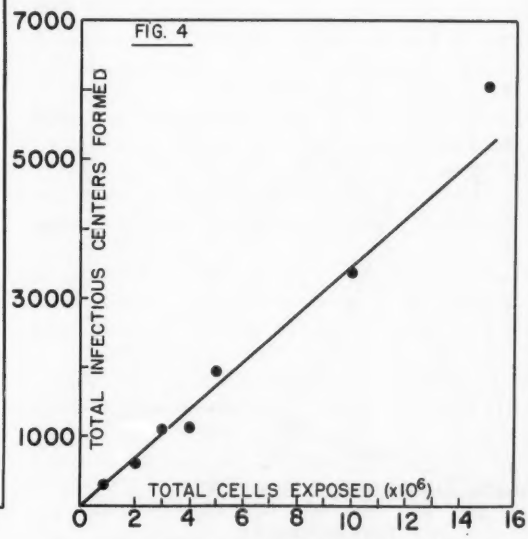

FIG. 4. Relationship between the number of infectious centers formed in "L" cell-Mengo RNA mixtures and the number of cells employed. Incubation was in 1.0 ml RNA for 15 minutes at 37°C.

cells with a standard virus suspension in the various media—the mixtures were diluted with 2× Eagle's in Earle's medium containing sufficient anti-Mengo gamma globulin to inactivate unabsorbed virus. The results are shown in table 3. Any increase in sodium chloride concentration to levels higher than physiological level virtually abolished cell-virus interaction. Although the effect of sucrose was somewhat less dramatic, increasing concentrations of this solute in the medium in which cells and virus were incubated sharply inhibited the formation of infectious centers. More detailed studies of the Mengo virus-"L" cell system have shown that the primary effect of these solutions of high osmolarity is on the initial virus-cell interaction. That is, they inhibit attachment of the virus to the cell.

Thermal Inactivation of Infectious Mengo RNA. A number of investigators have reported on the lability of infectious viral RNA preparations. The results of a study of the rate of inactivation of Mengo RNA in 0.14-M PBS, 0.64-M NaCl. and 0.7-M sucrose in PBS are illustrated in Fig. 2. Solutions of RNA in these three diluents were incubated at 37 C. At intervals, 1-ml aliquots were removed and tested for residual activity by their ability to form infectious centers in 10^6 cells during a 15-min incubation at 37 C. Early estimates of the half-life of RNA were 12–24 min. The addition of versene to a final concentration of 10^{-3} or 10^{-4} M increased the half-life by a factor of 4–5, suggesting that heavy metal contaminants catalyze the decay of the active molecule. Important to subsequent discussions is the fact that statistical analysis of the data makes it clear that there are no significant differences in the rates of thermal inactivation in the three diluents examined.

Relationship Between Infectious Centers Formed and Concentration of RNA. The number of infectious centers formed in a fixed number of "L" cells is proportional to the concentration of RNA over the range 5 μg to 1 mg per milliliter. Fig. 3 illustrates clearly the inverse proportionality between plaques formed and the dilution of RNA.

Another characteristic of the system is illustrated by this figure. The number of infectious centers produced in a fixed number of cells depends only upon the concentration of RNA. The number is independent of the volume of the RNA solution in which the cells are incubated—that is, independent of the total number of infectious units present. In Fig. 2, the number of infectious centers formed in 10^6 cells incubated in 1-ml and 2-ml aliquots of RNA solutions of varying concentrations is shown. There is no significant difference in the slopes of the two lines. More recently, we have noted that identical straight lines are obtained by using 10^6 cells in 0.5-ml, 1.0-ml, and 2.0-ml aliquots of RNA solutions.

It appears that the uptake of RNA may be a case of concentration-dependent diffusion across the cellular membrane. If this is so, then doubling the number of cells incubated with a fixed volume of RNA solution should double the number of infectious centers formed. This is, in fact, the case. The number of infectious centers formed in 1.0 ml of RNA solution is directly proportional

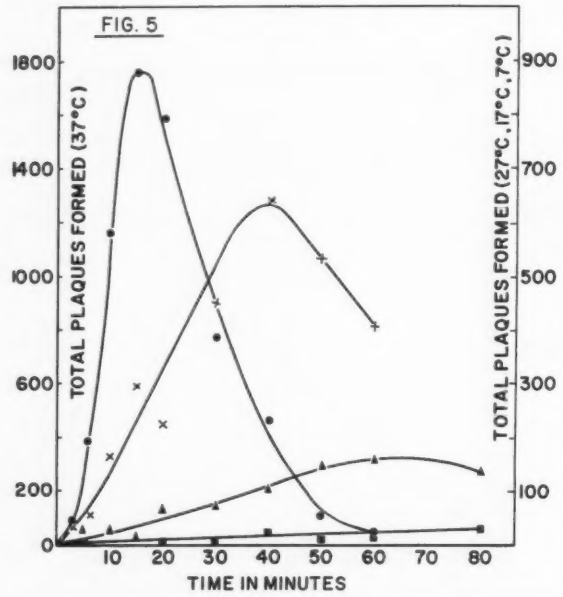

FIG. 5. Effect of temperature on the formation of infectious centers in "L" cells incubated with Mengo RNA. 10^6 cells incubated in 1.0 milliliter of RNA solution. Diluent = 0.7 molar sucrose in 0.14 molar PBS. ● = 37°C; X = 27°C; ▲ = 17°C; ■ = 7°C.

to the number of cells used over the range 10^6 to 2×10^7 cells. This finding is illustrated in Fig. 4.

It follows, from these considerations, that we can have no absolute measure of the infectivity of an RNA preparation, unless we can be sure of having absorbed all, or at least a known amount, of the nucleic acid from the solution. Perhaps the only way in which the total number of infectious units in an RNA preparation could be determined would be to measure, simultaneously, the number of infectious centers formed and the amount of RNA taken up by the cells. Preliminary experiments in our laboratory make it quite clear that the amount of RNA taken up by the cells under our conditions of assay is small—probably less than 1% of the total RNA present. An accurate estimate would almost certainly require the use of isotopically labeled RNA.

Effect of Temperature on Formation of Infectious Centers. The rate at which infectious centers are established is markedly temperature-dependent. The production of infectious centers at various temperatures is illustrated in Fig. 5. At 37 C, a maximum is reached after incubation for 15 min. When incubation is continued longer, there is a rapid decrease in the number of infectious centers. This decrease probably reflects a loss of viability of the cells. At 27 C and at 17 C, maximal production of infectious centers is seen at approximately 40 and 60

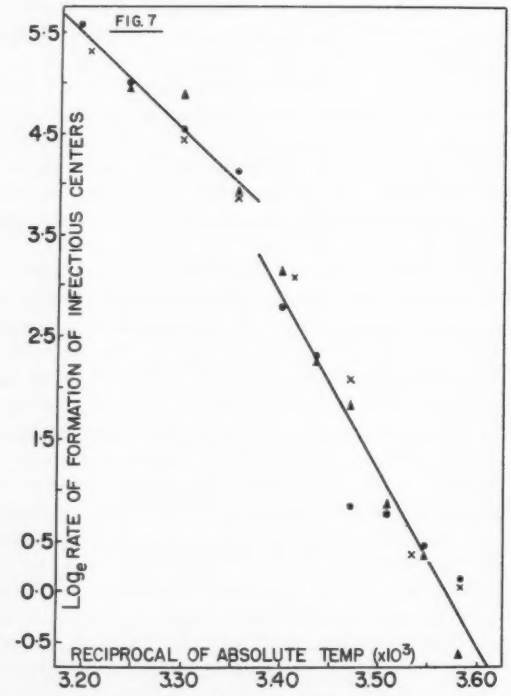

FIG. 6. Rate of formation of infectious centers in "L" cell-Mengo RNA mixture at 37°C. 2×10^6 cells were incubated in 1.0-milliliter volumes of 0.7 molar sucrose/PBS containing 50 μg RNA.

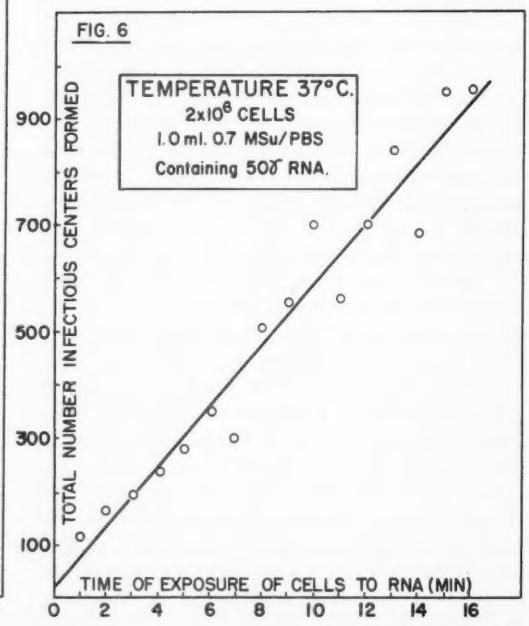

FIG. 7. Arrhenius plot relating the rate of formation of infectious centers in "L" cell-RNA mixture (expressed as logarithms to the base e) and the reciprocal of the absolute temperature.

min, respectively. The rate at 27 C is about one-fifth that at 37 C. Similar large reductions in the rate occur at 17 C and at 7 C. The results of a more careful examination of the rate of formation of infectious centers at 37 C are shown in Fig. 6. The formation is rapid and linear with time, during the first 15 min, at the end of which it levels off to a sharp maximum, then declining rapidly.

The effect of temperature on these reaction rates is adequately described by the Arrhenius formulation for "irreversible" first-order reactions,

$$\ln \frac{K_2}{K_1} = \frac{A}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right),$$

in which K_1 and K_2 are the velocity constants at absolute temperatures T_1 and T_2, respectively, R is the gas constant; and A is a constant characteristic of the system. An Arrhenius plot relating the rate of infectious center-formation (expressed as logarithm to the base e) to the reciprocal of the absolute temperature is shown in Fig. 7. The data used for this plot were corrected for the temperature-dependent effect of viscosity on collision frequency. The figure is actually composed of two straight lines, one covering the range 5 C–25 C, and the other from 25 C to 40 C. The values of the critical thermal increment or temperature characteristic, μ, calculated from the expression $\mu/2 = A/R$ (17), are approximately 20,000 and 30,000 calories for the upper and lower temperature ranges, respectively. This suggests that a high energy of activation is involved in the sequence of events during which infectious RNA meets, penetrates, and becomes stabilized in the cell as a replicating unit.

DISCUSSION

The data suggest that the mode of action of media of high osmolarity is on the cell, rather than on the RNA. Although RNA of high molecular weight is insoluble in 1-M NaCl at 0 C (18), it is completely soluble in this solution at 37 C. The RNA is soluble in solutions of sucrose as concentrated as 1.2 M. Hence, under the conditions of the reaction of cells and RNA, insolubility of RNA in the suspending medium can be excluded as part of the mechanism of the increased efficacy of establishing infection. Preliminary studies of the uptake of P^{32}-labeled RNA by "L" cells indicate that the cells do not absorb more RNA in the optimal sucrose or NaCl solutions than they do in 0.14-M PBS. Finally, the stability of RNA at 37 C is essentially the same in 0.14-M PBS, 0.65-M NaCl, and 0.7-M sucrose in PBS.

These observations strongly suggest that the hypertonic saline and sucrose solutions act by modifying the cell in some way, thereby increasing its receptivity or stabilizing capacity for the infectious RNA. Cellular ribonucleases may be inhibited by the high intracellular electrolytic concentration produced by dehydration. Crystalline ribonuclease has been shown to be inhibited by solutions of high ionic strength (19). Another possible mechanism is suggested by the demonstration, in our laboratory, that the infectivity of Mengo RNA is abolished or sharply inhibited by several ribonuclease-free proteins, when nucleic acid and protein are mixed in solutions of physiological ionic strength. At high ionic strengths, inhibition does not occur. It is possible that a hypertonic intracellular environment prevents non-specific immobilization of the viral RNA by cytoplasmic proteins.

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Biological and biochemical basis for cell injury by animal viruses[1]

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EXPERIMENTS reviewed by other essayists in this symposium (1, 2) have demonstrated clearly that viral nucleic acid is essential for initiation of the synthetic processes responsible for formation of mature viral particles, and that the information brought into a susceptible host cell by nucleic acids may direct the formation of new enzymes for synthesis of viral materials. These events lead to production of infectious viral particles, but the question may be posed, "Do they also directly initiate the injury of infected cells?"

One may postulate that viral infection causes major cell injury by two basic mechanisms.

1. Incorporation of infectious viral material (i.e., nucleic acid and possibly some protein fragments) redirect the host cells' metabolic processes to synthesize new materials for viral production, rather than for manufacture of important cell materials. This leads to: either *a*) injury and possible death of the infected cell resulting from the failure to replenish essential cell components; or *b*) damage of the cell because of the development of inclusion bodies. Inclusion bodies may be formed from either newly synthesized viral materials (viral particles or viral precursors made in excess of the quantity required for development of infectious virus) or damaged host-cell substances.

2. Large numbers of mature viral particles or toxin-like substances formed in viral infected cells may induce marked cytopathic changes in cells without concomitant viral multiplication.

The actual basis for cell injury probably differs in various viral infections. Furthermore, cell damage may be instigated by a combination of the mechanisms suggested, rather than one or the other. The evidence available from several experimental infections will be examined to indicate the mechanism responsible for cell injury in each example. A comprehensive review of the literature will not be attempted, but rather pertinent data to illustrate concepts will be described.[3]

INCORPORATION OF INFECTIOUS MATERIALS AND REDIRECTION OF HOST CELLS' SYNTHETIC PROCESSES

It has been generally considered that injury of virus-infected cells occurs because the cells' essential metabolic processes are redirected to synthesize viral materials. In effect, although this is a tempting hypothesis, there is little direct evidence to substantiate it. Infection usually results in cessation of cell division, although, in some instances (as with influenza and Newcastle disease viruses), one further mitosis may be completed after infection (3). Another notable exception to this generality occurs with cells infected with tumor-inducing viruses (4). However, even though infected cells in general do not replicate, failure of cell division, per se, does not necessarily result in severe injury and death of affected cells.

Detailed cytologic and cytochemical sequential studies of poliovirus (5, 6) or adenovirus-infected (7–9) cells clearly demonstrate that cell damage accompanies viral multiplication. With poliovirus, early inclusion-body formation was followed by death and lysis of the infected cell (5, 6). The mechanism underlying these events is unknown. With adenovirus infection, progressively more prominent intranuclear inclusion bodies developed, with time, but cell lysis did not occur (7–10). The basis for these pathologic changes in adenovirus-infected cells now will be considered.

Some important characteristics of adenoviruses will be summarized, for they are necessary to an understanding of the mechanism by which cell damage is incurred. These viruses multiply within nuclei of susceptible cells (8, 9, 11–13), and cells are not lysed as a result of infection (10). Moreover, infected cells continue to carry on active metabolic functions even after extensive cell

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[3] Investigations from our laboratory will only by summarized; the detailed data have either been published previously, and the bibliographical references are given, or the studies are in the process of publication elsewhere.

damage has occurred (10, 14). Not only do infected cells remain intact after viral multiplication and cell injury, but viral particles do not dissociate readily from cells in which they are synthesized (15). Present evidence, although not conclusive, indicates that deoxyribonucleic acid (DNA) is the nucleic acid constituent of adenoviruses (16).

Adenoviruses instigate a particularly striking series of cellular changes during the course of viral infection (7–9). The specific damage occurs in the nucleus, the site of viral synthesis. The earliest change noted is a rearrangement of the chromatin material. This is followed by enlargement of the nucleus; a gradual increase in quantity of basophilic material, which is, at first, granular in appearance; and accumulation of basophilic material in the central portion of the nucleus, the so-called central mass. Finally, with types 3, 4, and 7, large basophilic, crystal-like structures are formed; the nucleus becomes larger; and the central mass becomes more prominent (7–9). Careful studies by several investigators have demonstrated these crystal-like bodies to be composed of spherical viral particles arranged in a crystalline lattice (11–13). It should be emphasized that the nucleus is relatively intact, despite presence of large numbers of viral particles and the accumulation of masses of basophilic material. Cytochemical studies demonstrated that the crystalline structures and the basophilic central mass contained large quantities of DNA (7). With the use of fluorescent-antibody techniques it was observed, however, that although the crystals were composed of specific viral antigens, the central basophilic mass, which contained a large amount of DNA, comprised a relatively small amount of the specific antigen (8, 9). Thus, although DNA may accumulate, much of the nucleic acid is not contained in viral particles or associated with specific antigenic viral proteins.

Experiments were devised to investigate the biochemical basis for the cellular alterations described. Extractions of nucleic acids from control cells and infected cells readily showed the accumulated nucleic acid to be a consequence of increased synthesis of DNA, rather than of disorganization or rearrangement of chromatin material (16). Subsequent experiments, in which infected cells were extracted in series with 0.15-M NaCl followed by water or 1-M NaCl, suggested that at least two species of DNA nucleoprotein existed in infected HeLa cells: one was extracted in 0.15-M NaCl; the second was not soluble in 0.15-M NaCl, but was in water or in 1.0- –2.0-M NaCl. The first, that soluble in 0.15-M NaCl, was the smaller in amount, and will subsequently be referred to as "saline-soluble DNA." The second, that soluble in water or hypertonic NaCl, comprised the majority of cellular DNA, and will be referred to as "water-soluble DNA." Very little saline-soluble DNA was obtained from uninfected cells. In contrast, adenovirus infection effected a marked accumulation of saline-soluble DNA (approximately a 4.5-fold increase), but a relatively small increase in water-soluble DNA. A small increase in total RNA was also consistently measured in virus-infected cells. Approximately a twofold increase in protein occurred in infected cells; the increase was of the same magnitude in fractions extracted with either 0.15-M NaCl or water (16). It should be emphasized that more than 90% of infectious virus was present in the 0.15-M NaCl extract. However, ultracentrifugal studies showed that the vast majority of DNA present in this fraction was not incorporated into viral particles (16).

These data suggested that two species of DNA were present in HeLa cells, and that increased synthesis of the saline-soluble fraction occurred in adenovirus-infected cells. Experiments with P^{32}-labeled inorganic phosphate confirmed this hypothesis. A marked increase occurred in the specific activity of saline-soluble DNA from infected cells, but no increase in the specific activity of water-soluble DNA was noted (17).

Experiments were next designed to determine whether the saline-soluble DNA, synthesized as a result of viral infection, was derived from host material or from precursors contained in the media. Cell components were labeled with P^{32}-labeled phosphate or C^{14}-labeled formate for 48 hr before infection. Prior to viral inoculation, isotope was removed; cultures were washed; and maintenance medium without isotope was added along with virus. Specific activities of the nucleic acids were determined 24 hr after infection. Under these conditions, no increase in specific activity of the saline-soluble DNA was measured. Indeed, a consistent decrease of 14–25% occurred in specific activity of the saline-soluble DNA, as compared with DNA from uninfected cells. It is important to point out that this decrease in specific activity was not nearly so great as the increase in specific activity when isotope was added with, or 21–23 hr after, virus, or was it of the same magnitude as the measured increase in saline-soluble DNA in infected cells. These data imply that precursors of the DNA were obtained from the precursor pool of the cell (17) as well as from the medium.

The question was next posed whether synthesis of saline-soluble DNA after viral infection related temporally to viral multiplication? The DNA synthesis was measured by both P^{32} specific activity and total saline-soluble DNA. The eclipse period of the initial cycle of multiplication of the type 4 adenovirus was 14–15 hr, as shown in previous studies (15). Saline-soluble DNA (measured chemically) began to increase in infected cells at approximately the same time, between 14 and 16 hr after infection. In striking contrast, the increased incorporation of P^{32} into this species of DNA commenced approximately 12 hr after infection—2–3 hr before total saline-soluble DNA or virus increase could be detected. Experiments with C^{14}-labeled formate, in place of P^{32}-labeled phosphate, confirmed this finding (17). These data imply that saline-soluble DNA and viral synthesis are related temporally, and that synthesis of saline-soluble DNA is initiated before mature infectious virus can be detected. It can be postulated, on the basis of

this evidence, that saline-soluble DNA may be a precursor of virus, and is fabricated into the viral particles. Proof of this hypothesis will depend on preparation of highly purified virus, and determination of its base structure and radioactive specific activity, as compared with the saline-soluble DNA isolated from infected cells.

Evidence obtained suggested that adenovirus-infected HeLa cells contained two different molecular species of DNA. Possibly, the difference in solubility characteristics may have been chiefly due to differences in basic proteins with which each species of DNA complexed. To determine if actual differences in the structure of the two species of DNA existed, quantitative analyses of the nucleotides of each were made. The DNA's were hydrolyzed enzymatically, and the nucleotides separated by column chromatography. Unfortunately, by the methods employed, it was not possible to obtain sufficient saline-soluble DNA from uninfected cells to give reliable results. Therefore, the saline-soluble DNA from infected cells was compared only with water-soluble DNA from infected and control cells. There was a striking difference between the saline-soluble DNA from infected cells and the DNA from uninfected cells (16). The difference was most marked by an increase in the level of guanylic acid and a comparative decrease in the level of adenylic acid. Water-soluble DNA from uninfected and infected cells had equal pairing of adenine to thymine and of guanine to cytosine—evidence to imply that the structure of this DNA is consistent with the Watson-Crick hypothesis, according to which DNA is a double-stranded molecule (18). In sharp contrast, however, molar ratios of the saline-soluble DNA from infected cells were not compatible with complementary pairing of adenine to thymine, and probably not of cytosine to guanine. These data imply that the saline-soluble DNA is a molecular species different from the normal host DNA, and that the saline-soluble DNA, because of an absence of base-pairing, does not fit the Watson-Crick model. Moreover, these data suggest that the saline-soluble DNA may be an example of a DNA of unusual structure: perhaps double-stranded, with loop side-chains (19); triple-stranded; or, possibly, a single-stranded polymer (20). Additional evidence to be summarized tends to support the last of the three alternatives.

Incorporation of P^{32} into nucleotides of saline-soluble DNA also provides further evidence that the saline-soluble DNA from infected cells is different and is possibly a new and novel DNA, at least so far as the HeLa cell is concerned. Although, there was a markedly increased P^{32}-incorporation into all nucleotides, the relatively increased incorporation of P^{32} into deoxyguanylic acid of saline-soluble DNA from infected cells was strikingly even greater (17).

Experiments were also performed using, as precursors, adenine-8-C^{14} or C^{14}-labeled sodium formate. The saline-soluble DNA was extracted from uninfected and multiply infected HeLa cells, hydrolyzed to bases with formic acid and separated by two-dimensional paper chromatography. As previously demonstrated by Salzman and Sebring (21), adenine-8-C^{14} was incorporated into the purines of DNA in HeLa cells. The specific activity of adenine and guanine was increased threefold and sevenfold, respectively, in saline-soluble DNA of infected cells. For emphasis, these findings may be expressed in another manner: the specific activity of guanine was approximately twice that of adenine in DNA from infected cells, whereas the specific activities of the two purine bases were of the same order in DNA from uninfected cells.

Experiments in which C^{14} labeled sodium formate was employed as precursor demonstrated incorporation of the precursor into thymine as well as into the purine bases. These studies further indicated that the specific activity of bases from saline-soluble DNA was markedly increased, and that incorporation of one-carbon fragments into guanine as compared with other bases in the saline-soluble DNA from infected cells was greatly augmented.

These data substantiate the notion, proposed earlier, that the saline-soluble DNA is a molecular species of nucleic acid, distinct and different from the bulk of DNA present in either uninfected or adenovirus-infected cells. Furthermore, the results presented suggest that the saline-soluble DNA from infected cells is novel and different from the DNA that is extracted from uninfected cells with 0.15-M NaCl.

To investigate the structure further, the saline-soluble and water-soluble DNA's from infected cells were extracted by the phenol method, as modified by Kirby (22). This procedure yielded a preparation essentially free of protein, but containing considerable RNA. Efforts were made to remove RNA by incubation with ribonuclease. This proved to work successfully with the water-soluble DNA. It was striking, however, that, in the saline-soluble DNA preparation, less than 10% of the RNA was hydrolyzed even when incubated 2–3 hr with a large excess of ribonuclease. Of further significance concerning the structure of this DNA molecule was the finding that, after the DNA-RNA complex was heated at 60 C for 60 min, treated with ribonuclease, and dialyzed, no RNA could be detected by the orcinol reaction (23).

These data suggest that the saline-soluble DNA molecule has many free, reacting groups with which RNA can complex firmly. Furthermore, these results present further evidence which implies that the saline-soluble DNA, synthesized within virus-infected cells, has a molecular structure different from that which is considered to be the host-cell DNA, the water-soluble DNA fraction.

Additional evidence bearing upon this postulate was obtained by a study of the hyperchromic effect produced by heat denaturation of DNA freed from proteins and RNA. Purified (phenol-extracted, heated at 60 C for 60 min, and ribonuclease-treated) water-soluble and saline-soluble DNA from infected cells were dialyzed against phosphate-buffered 0.1-M NaCl and heated at 100 C for 15 min. The water-soluble DNA—like a thymus DNA

control treated in the same manner—was denatured by this treatment, and showed a marked hyperchromic effect. In contrast, only a very minimal increase in ultraviolet absorbence followed heating of the saline-soluble DNA. These results further indicate that there is a marked difference in the structure of the two DNA molecules. The absence of a marked hyperchromic effect, after heating of the saline-soluble DNA, suggests that it does not have a double-helical structure, in which the strands are joined by hydrogen-bonding. However, additional evidence must be obtained by using highly purified DNA before one can be completely certain of the structure of saline-soluble DNA.

The experiments described emphasize a mechanism by which cells manifest injury effected by a viral infection. The striking intranuclear structural changes observed are the visual reflection of the biochemical events induced by adenovirus infection. Indeed, specifically these changes probably result from the synthesis and the marked accumulation of DNA and protein, which are new and novel substances.

INJURY OF CELLS BY VIRUS OR VIRAL PRODUCTS, WITHOUT VIRAL MULTIPLICATION

It seems apparent that the picture thus far described is not the entire basis for cellular injury by viruses. Indeed, other factors are also involved in the mechanism of production of cytopathologic changes by adenoviruses (7, 24–26). As mentioned earlier in this discussion, there is abundant evidence to demonstrate that, in the absence of complete viral multiplication, viruses may injure and even kill susceptible cells. The most striking example of this phenomenon is "lysis from without" by the *E. coli* phages (27). This may be accomplished not only by infectious phage, but also by the phage-coats devoid of nucleic acid (28). There are now numerous examples of infectious animal viruses that induce similar effects, the best described being those of influenza (29–33) and Newcastle disease (34), on susceptible cells, and of lysis of certain red blood cells by the viruses of mumps (35) and Newcastle disease (36–38). The biochemical basis for injury of host cells with any of these agents is not yet clear. However, suggestive evidence has been obtained with certain model systems in vitro.

It was demonstrated that influenza viruses combine with polymorphonuclear leukocytes by reactions similar to association of these agents with susceptible host cells in vivo (39). Merchant and Morgan showed that this reaction markedly reducing the ability of the white blood cells to phagocytize bacteria (40). Investigation of the basis for this phenomenon yielded several pertinent findings (41, 42). Influenza B virus markedly reduced the evolution of CO_2 from bicarbonate buffer by polymorphonuclear leukocytes when glucose was used as substrate. Balance-experiments demonstrated that the inhibition of CO_2 evolution resulted directly from decreased glucose utilization and lactic-acid production. It should be further noted that influenza viral particles did not lyse the leukocytes with which they reacted, but rather blocked the metabolic pathway of anaerobic glycolysis. Virus inhibited glycolysis when glucose or glucose-6-phosphate was employed as a carbohydrate source. However, when fructose-6-phosphate or fructose-1, 6-diphosphate was utilized as substrate, anaerobic glycolysis was not reduced by virus. These data suggest that the reaction of influenza virus with leukocytes induced a metabolic block between glucose-6-phosphate and fructose-6-phosphate in the anaerobic glycolytic pathway. This evidence suggests that virus affected the enzyme phosphohexoisomerase (41).

Further experiments indicated that influenza viruses inhibit the capacity of leukocytes to phagocytize particles through a similar mechanism (42). Phagocytosis was inhibited by influenza B virus when glucose or glucose-6-phosphate was used as substrate. However, when fructose-6-phosphate was the carbohydrate utilized, phagocytosis was not reduced (42). These data furnish the biochemical basis for a "physiologic injury," induced by a virus: a specific metabolic block of the Embden-Meyerhof pathway of anaerobic glycolysis.

As one final example of cellular injury by virus in the absence of viral multiplication, let us turn once more to the adenoviruses. In cells infected with this agent there is synthesized a specific viral antigen, which causes cytopathic changes of HeLa cells in the absence of viral multiplication (24–26). Indeed, the gross cytopathologic changes induced by this material (rounding and clumping of cells) are very similar to the injury caused by viral infection. Separation of the toxin-like material from the viral particle is a unique virologic finding (24, 25). The "toxin" has been shown to be antigenic, heat-stable, and largely protein in composition. By chromatography using DEAE-cellulose, one can separate, from a homogenate of virus-infected cells, toxin, infectious virus, and two complement-fixing antigens (43–45). At pH 7.2, toxin and one of the soluble complement-fixing components eluted as a single peak, at low ionic strength (0.08-M NaCl). These materials were separable from the soluble, complement-fixing antigen, which eluted at high electrolyte concentration (0.26-M NaCl). They were also separable from the infectious viral particles which eluted maximally from DEAE at 0.40-M NaCl. The toxin–complement-fixation (CF) peak, when chromatographic study was repeated, at pH 5.2, yielded two distinct components: toxin and the so-called early CF antigen. Only the toxin induced cytopathic alterations of cells in culture (44). An immunologic analysis of these fractions was carried out using neutralization, complement-fixation, agar-gel–diffusion, and immunoelectrophoretic techniques. From these experiments, in which the starting material was a homogenate of type 5-infected cells, the following data emerged: *1*) the purified early CF antigen reacted only with type 5-specific antibody or antibody prepared with the material which eluted at pH 7.2 with 0.08-M NaCl (it contained toxin and early CF antigen), but not with type 4, type 2, or late-peak CF-component antibodies;

thus, it is type-specific; *2*) toxin reacted with antibodies against all types of adenovirus; it is a group-specific antigen; *3*) the late CF antigen reacted with antibodies to types 2, 4, and 5 viruses as well as with the homologous antibody, but did not react with antibody against the early CF component; it is the group-soluble complement-fixing antigen; *4*) infectious virus reacted with, and was neutralized by, type-specific antibody, as well as by antibody against the purified early CF antigen (44, 45). It may be deduced from these results that the early CF antigen and the infectious virus are both type-specific; that the toxin and the late-eluting CF antigen are group-specific; and that some or all of the soluble antigens identified are probably precursors of mature viral particles. The antigens separable from the mature viral particles are probably produced in great excess, thereby accumulating in the infected cells.

CONCLUSIONS

One may postulate, from the evidence cited, that injury of adenovirus-infected cells is the direct result of an uncontrolled synthesis of viral components. The protein, formed in excess in the virus-infected cells, is similar to the protein antigens present in the infectious viral particles. Preliminary investigation suggests that the adenoviruses are DNA viruses (17). It is hypothesized that the new and novel saline-soluble DNA, obtained from infected cells, is identical with the nucleic acid present in viral particles. Because infected cells are not lysed, the DNA and protein continue to be synthesized in uncontrolled manner in great excess of that required for fabrication of mature virus. These materials that accumulate in infected cells may, then, be termed "surplus" DNA and protein. The accumulation of "surplus" DNA within the nucleus forms inclusion bodies which are the recognized hallmark of adenovirus-infected cells. The large amount of "surplus" protein that is synthesized as a result of infection contains biologically active protein, which is a toxin-like substance that causes further cellular injury and is noted as a rounding and clumping of the cells—the characteristic, gross, cytopathic effect observed.

Thus, with adenoviruses, we note that two mechanisms are responsible for cellular injury: *1*) synthesis and accumulation of specific materials, thereby forming inclusion bodies; and *2*) direct injury of a cell by a toxin-like material. These are bases by which animal viruses can injure host cells—either alone or in combination. It is not implied that these are the only means by which viruses inconvenience cells; rather, the examples cited are models of the mechanisms by which a virus-infected cell may be damaged.

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Bacteriophage with single-stranded deoxyribonucleic acid

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THE DISCOVERY of a new category of virus (1), containing as its apparent genetic material, a single-stranded form of deoxyribonucleic acid (DNA) (2), has enlarged our horizons, and has raised numerous questions and numerous research opportunities concerning viral function and replication. To date, despite some suggestive evidence in other areas, viruses of this class are known only among the bacteriophage and, indeed, only the *E. coli* phage, ϕX 174, has been convincingly demonstrated to be of this type, although it is most likely that the related bacteriophage, S13, is similar (3).

All the work I will consider, then, concerns the prototype virus, ϕX 174. This is a virulent bacteriophage which grows within, and invariably lyses certain strains of, colon bacteria. The virus can be collected from lysates, and, after extensive purification, can be obtained in ultracentrifugally pure form. Light-scattering studies of the virus indicate a particle weight of 6.2×10^6. Thus, it compares in size with the smaller plant or animal viruses, and is much smaller than a bacteriophage like T2, which has a particle weight of 240 million. Elegant electron micrographs of ϕX, taken by Cecil Hall and associates (4), indicate that it has a considerable degree of substructure. It appears to have pentagonal symmetry, and the pattern observed can be interpreted either as a pentagonal dodecahedron, with a protuberance on each face, or as an icosahedron, with a bulge at each vertex. In either case, one might expect this surface structure to be formed of identical protein subunits—12, 24, or 60, in number.

Dr. Carusi, in our laboratory, has recently been able to decompose, in alkali, plus detergent, the protein coat into what appears to be a subunit (Fig. 1) of sedimentation coefficient about 5s. Although a molecular weight for this particle has not yet been obtained, this is about the sedimentation rate to be expected for a $\frac{1}{60}$ piece, which would be of a molecular weight of about 75,000.

This 5s component is still antigenic. It reacts with the inactivating antibody in antisera to ϕX bacteriophage. Gel-diffusion tests have previously indicated (according to U. Rolfe, in unpublished data) the presence of two antigenic sites on the ϕX particle. One of these appears to be, at least, a portion of the 5s protein, and the blocking of such sites by antibody appears to inactivate the virus. In addition, as can be shown by exhaustion of anti-ϕX sera against 5s preparations, there is a second antigenic site and a corresponding antibody. This second antibody acts at low dilution to precipitate ϕX, but does not inactivate it at the usual levels of antiserum employed. It is, conceivably, an antigen against some contour formed by aggregation of the 5s material into the virus coat. Additionally, antisera to purified 5s protein will inactivate ϕX.

So far, no protein other than the 5s unit has been obtained from the protein coat, and this unit must constitute at least 80% of the coat.

The virus contains 25.5% DNA by weight. It is devoid of RNA. The DNA content is, thus, about 1.7×10^6. When isolated from ϕX by the phenol method (other methods have given similar results), the DNA obtained has a molecular weight of 1.7×10^6, indicating the presence of 1 molecule per particle. This nucleic acid content is similar to the RNA content of the small RNA-containing viruses, such as the turnip-yellow mosaic or the poliomyelitis (5) virus.

A variety of lines of physicochemical evidence was used to establish that this DNA has unusual properties and is, indeed, single-stranded. Studies of the influence of ionic strength and temperature on configuration and on hypochromicity indicated that this was a flexible molecule, and one that lacked the usual manifestations of an ordered, hydrogen-bonded structure. Studies of the kinetic aspects of deoxyribonuclease digestion fitted well the expectation for a single-stranded structure. The ability of this DNA to react with formaldehyde was demonstrated spectrophotometrically even when within the virus. This result again indicated the absence of a regularly hydrogen-bonded structure, and excluded possibilities of artifact. Finally, the nucleotide composition was shown to be clearly noncomplementary.

The conclusion that this virus contains a single-stranded DNA provided a ready explanation for the concurrent observation by Tessman (3), namely that the efficiency of inactivation of ϕX 174 by decay of incorporated P^{32} is 1.0 per P^{32} disintegration rather than 0.1, as had been observed with all other bacteriophage. Tessman also, more recently, neatly confirmed this conclusion by an elegant genetic experiment (6). As was

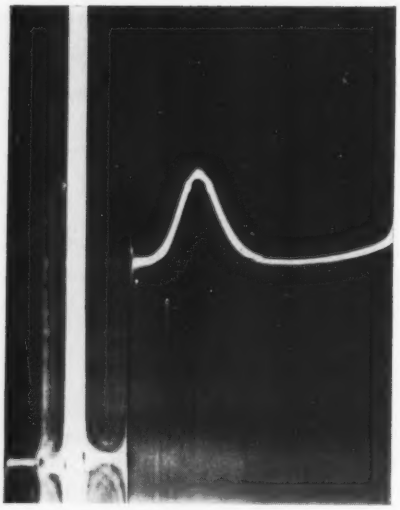


FIG. 1. Sedimentation pattern of 5s protein from ϕX coat, frame taken after 40 minutes at 57,040 rpm, 20°C.

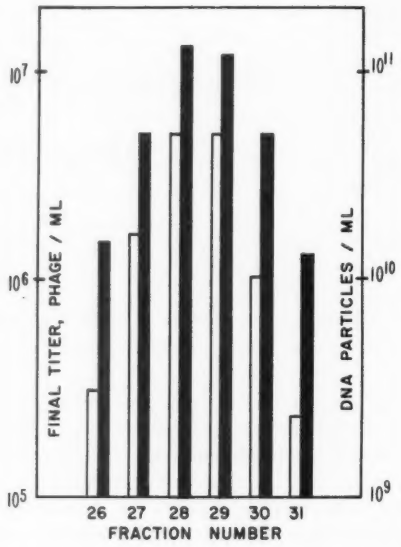


FIG. 2. Infectivity and particle concentration of samples from a cesium chloride density gradient band of ϕX-DNA; mean density of the band is 1.72 gm/cm^3. Biological activity is shown by open bars, particle concentration, calculated from the ultraviolet absorption, by solid bars.

first demonstrated with the tobacco mosaic virus by Mundry and Gierer (7), nitrous acid is a potent mutagen that appears to act by deamination of the purine and pyrimidine rings of nucleic acid. Nitrous acid is also mutagenic for bacteriophage. With bacteriophage T4, it has been observed that, among the phage surviving the treatment, many are heterozygotes (8), that is, in single infection, they give rise to cultures with equivalent numbers of mutant and wild-type virus particles. Such heterozygotes are presumed to arise from deamination of one strand of the two-stranded DNA of T4, thus producing a particle with one mutant strand and one wild-type strand, each of which contribute to the progeny. Now, as ϕX 174 has but a single strand of DNA, such heterozygotes should not be possible; indeed, Tessman showed that the mutants, produced in ϕX 174 by the action of nitrous acid, are all homozygous, which is in agreement with the expectation.

Having but one strand of DNA, it would appear to be potentially feasible to produce infection of bacteria with the free nucleic acid, if there were means to facilitate its entry into the cell. To achieve this, attempts have been made, with some success, to obtain infection of bacterial protoplasts with ϕX DNA (9).

The protoplasts are made by the lysozyme-plus-versene method of Fraser et al. (10). Protoplasts of the usual host, *E. coli* strain C, or of the resistant mutant, C/ϕ, or of different strains of *E. coli*—B or K12, which do not usually serve as hosts for this virus—are all equally effective. The use of protoplasts of resistant strains has advantages in excluding the possibility that the virus particles produced are a consequence of infection of some residual whole cells by some residual phage in the viral nucleic acid preparation, although the nucleic acid preparations are free from any demonstrable infective virus particles. Whole ϕX particles will not infect these protoplasts, and ϕX DNA will not infect whole cells.

If ϕX DNA is added to a suspension of properly prepared protoplasts, and nutrients are supplied, infective centers are formed (which plate upon the usual host, *E. coli* strain C) and, after a latent period, free phage begins to appear in the medium. Quite respectable titers of phage, 10^9 per milliliter or higher, can be obtained from this system. The number of infective centers obtained is not linearly related to the number of DNA molecules added, so that whereas, with high concentrations of DNA, only one infective center is formed per 10^5 DNA molecules, at low concentrations, the efficacy of infection rises to 10% or higher, and a net synthesis of viral nucleic acid is obtained.

That the infection here is actually owing to DNA is demonstrated by the susceptibility of the infective element to deoxyribonuclease, by the infection of protoplasts of normally immune strains, by the total lack of any infectivity upon whole cells of the normal host, and by the congruence of the biological activity of these preparations (Fig. 2) with the ultraviolet absorption of the ϕX DNA, when this DNA is banded in a cesium chloride density gradient (11).

This system, with which infection can be obtained with the free nucleic acid, has many potential applications. It is being used to study the course of synthesis of the ϕX nucleic acid during the infective process.

It is also possible to obtain subviral agents, other than the free ϕX DNA, which are infective for protoplasts, but they are no more infective per DNA complement.

FIG. 3. Equilibrium positions of ϕX (left) and heated ϕX (right) particles in a CsCl density gradient.

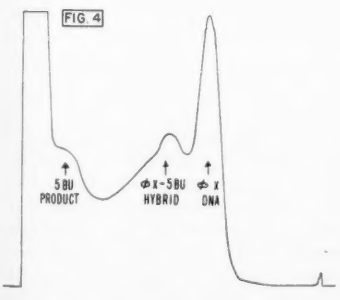
If virus particles are heated for 5 minutes at 70 C, a discrete reaction takes place. A new particle is formed of sedimentation rate about 45s. A small amount of free DNA is liberated. Further heating at 70 C does not alter this pattern. The 45s particles still retain DNA, as indicated by ultraviolet absorption, but this DNA is now exposed to deoxyribonuclease. If the particles are treated with deoxyribonuclease, the sedimentation rate rises to about 70s. Changing ionic conditions also affect the sedimentation rate of these particles; they act as though the virus particle had popped open and its DNA were now streaming out. Indeed, electronmicrographs, by Hall and colleagues, indicate that this is exactly what has happened (12).

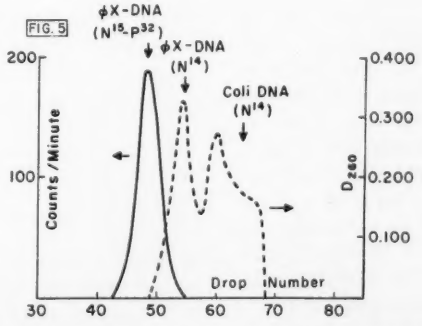
Such heated preparations are infective upon protoplasts. (This was first observed by Tessman, according to a personal communication.) These particles band at a slightly greater density than does native virus in the cesium chloride density gradient, so they can be separated from the traces of intact virus that remain (Fig. 3). They appear to have lost some protein, but clearly retain much ot it, as they band far away from DNA. Thus, the particles could be isolated from this band, and their infectivity to protoplasts assayed. They are infective to protoplasts, but not more so than is the free DNA.

Perhaps the most intriguing question concerning the single-strand DNA virus is that of its replication. How does a single-stranded nucleic acid replicate? It is interesting that, as cooperative experiments with Dr. Arthur Kornberg and his group have shown, if ϕX DNA is used as a primer with the DNA polymerase, a product having the viscosity and hypochromicity of a two-stranded DNA is formed (13), and this, then, goes on to make more two-stranded DNA. This result was demonstrated in a clear way by the use of ϕX DNA as the primer, and the substitution of 5-bromouracildeoxyriboside - triphosphate for thymidine - triphosphate in the reaction mixture. In this way, the new strands containing 5-bromouracil are heavier than the old ones, and the first result expected, then, would be the formation of a hybrid DNA molecule, one strand containing thymine and one strand containing 5-bromouracil, which should band in the cesium chloride density gradient at about 1.74 density. The DNA molecules made subsequently from this hybrid would be 50% hybrid and 50% completely 5-bromouracil–substituted. Molecules of the latter type should band at a density of about 1.79. This was just the result obtained (Fig. 4) when ϕX DNA was used as a primer, and the polymerization allowed to proceed to somewhat more than double the amount of DNA.

This result immediately raised the question whether in vivo, the ϕX DNA forms a two-stranded structure during its replication. Of course, even if it did, this does not explain how it would ever get back to a single-stranded form, but the question is certainly of interest. Experiments have recently been undertaken to obtain, at least, some preliminary answers to this question.

The ϕX virus has been prepared labeled both for radioactivity with P^{32} and for density by complete substitution of N^{14} by N^{15}. The DNA of such particles is easily distinguished in density from normal N^{14}, ϕX DNA or from host DNA (Fig. 5). Cells have been infected with such phage; the infection stopped at 7–8 min; the cells centrifuged, washed, and lysed; and the lysate deproteinized with phenol. After some purification, carrier N^{14}, ϕX DNA is added to the deproteinized cellular extract, and this material is banded in an equilibrium cesium chloride density gradient in a lusteroid tube. The distribution of radioactivity and of ultraviolet absorption in the density gradient is examined





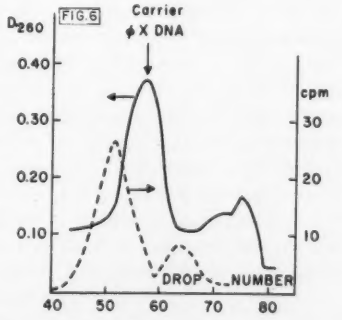


FIG. 4. Densitometer tracing of ultraviolet photograph of contents of ultracentrifuge cell containing ϕX DNA as a density marker and the products of synthesis when ϕX DNA is incubated with DNA polymerase and 5-bromouracil is incorporated; CsCl density gradient, at 44,770 rpm after 24 hours of centrifugation.

FIG. 5. Separation of N^{15}-ϕX-DNA (containing P^{32}), N^{14}-ϕX-DNA and E. coli DNA after banding in a CsCl density gradient.

FIG. 6. Radioactivity and ultraviolet absorption observed in alternate drops collected from a CsCl density gradient after 48 hours of centrifugation at 37,020 rpm at 2.5°C. The material banded in the gradient was a mixture of the DNA extracted at 6 minutes after infection from *E. Coli.* cells infected with N^{15} P^{32} labeled ϕX virus particles, and added N^{14} ϕX DNA, employed as a density marker (to be located by its ultraviolet absorption).

by flowing the solution, dropwise, through a hole punched into the bottom of the tube at the end of the centrifuge run, followed by analysis of each drop. The result of one such experiment (Fig. 6) is that much of the radioactivity is found just where single-stranded, N^{15}, ϕX DNA would be expected to band, relative to the carrier N^{14}, ϕX DNA located by its ultraviolet absorption. Since these experiments are carried out at a multiplicity of plaque-former per cell of considerably less than one, and since the number of infected cells was equal to the number of viable phage particles added, this DNA cannot have just come from cells wherein nothing is happening. Indeed, the amount of radioactive DNA recovered from the extracted cells is just what would be expected, knowing the fraction of viable particles in the phage preparation. However, a small band is also found at a second, lighter density. Now, it is rash to attempt to identify a substance by only its density. However, it is of interest that the band is at the density to be expected for a two-stranded molecule, to be composed of one N^{15}, ϕX strand and one N^{14} complementary strand.

The *E. coli* DNA bands at a density of 1.710. A complementary, two-stranded ϕX DNA would—from its adenine-to-guanine ratio—be expected to band at 1.702 (14, 15). A hypothetical hybrid—N^{15}, N^{14}, ϕX DNA—should band at 1.709. This would be, in the experiment, about 8 drops lighter than the carrier, N^{14}, ϕX DNA.

This experiment has been repeated with similar results. With increasing time after infection, more of the radioactivity appears in the lighter band. This mixed result is rather hard to interpret at present. It may be that the putative two-stranded form is not achieved until some time after infection. In any case, of course, its significance remains to be determined.

The availability of this unique virus, ϕX 174, thus appears to offer an unparalleled opportunity to probe deeply into the mode of replication of a single-stranded nucleic acid.

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NUTRITIONAL SIGNIFICANCE OF SELENIUM (FACTOR 3)[1,2]

Introduction

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SELENIUM, one of the rarer elements of our planet's surface, received its name from the moon. The Swedish chemist Berzelius discovered it in 1817 as a constant and faithful companion of tellurium which had been named after earth. In biochemical systems, companionship—and antagonism—of selenium with another element of the sixth group, namely sulfur, is of great importance. Sulfur and selenium are quite similar, and a rather perfect analogy is observed between their organic derivatives. This similarity makes it difficult to separate selenium analogues from sulfur compounds even by modern techniques of fractionation, a situation which is compounded further by the fact that selenite and also selenides readily react, in vitro and in vivo, to attach themselves unspecifically to all manner of sulfhydryl groups.

There is an elusive quality about the element: selenium dioxide and many organic derivatives are volatile. Most selenium compounds are also highly reactive, which makes the task of purification of natural organoselenium compounds even more arduous. The complications are best illustrated by the observation that not even the main low-molecular organoselenium compound found in seleniferous plants, of which the latter contain up to 1 % or more, has been obtained in pure form in spite of several long-lasting efforts over the past 30 years. However, it has been tentatively identified recently.

In animal tissues and in most nutrients, selenium occurs normally and physiologically in very small amounts. It is organically bound (as "Factor 3") and highly effective in the prevention and cure of a large variety of fatal deficiency diseases. The nutritional importance of Factor 3-selenium has been recognized only lately, and although we have a fair idea of the chemical properties of Factor 3-selenium as it occurs in nutrients and in the organism, the exact chemical structures of the active compounds remain to be determined.

Despite their companionship, sulfur and selenium frequently show a pronounced biochemical antagonism against each other in plants and microorganisms. This phenomenon may play a role in the development of enzootic Factor 3-selenium deficiency diseases in cattle and sheep. It is in these species that selenium, immediately after discovery of its physiological role, has started to find a wide and important field of practical application. Especially in New Zealand conditions for study and field trials of selenium responsive diseases are ideal, and Dr. W. J. Hartley, whose participation in this symposium is gratefully acknowledged, has made optimal use of these unique opportunities.

While it is likely that selenium, when given in large amounts, may travel with sulfur in its metabolic channels, available evidence suggests that it follows pathways of its own when supplied at small, physiological levels. These amounts are so minute that they usually escape determination by classical methods of analysis, but application of radioactivation analysis and of Se^{75} can overcome most of these obstacles. Thus, although there are technical difficulties to be overcome, many important and challenging biochemical problems have been opened up; not the least of them would be the clarification of the pathways of Factor 3-selenium assimilation, the identification of the active form of selenium within the organism, and the mode of action of Factor 3-selenium in intermediary metabolism.

[1] A symposium held in Atlantic City, N. J., April 13, 1961. Sponsored by the American Institute of Nutrition. Travel grants in support of this symposium were kindly supplied by Abbott Laboratories, Chicago, Ill.; Eli Lilly and Company, Indianapolis, Ind.; and the Nutrition Foundation, Inc., New York City.

[2] The participants in this Symposium express their thanks to Dr. E. L. Hove, the co-chairman, for his valuable assistance during the session.

Development and status of experimental work on Factor 3-selenium[1]

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THE OPPORTUNITY of presenting a summary report on the development and the current status of the Factor 3 project is much appreciated, particularly in view of the circumstance that most of this work has not been published as yet. From 1949 untill 1957 the project dealt mainly with attempts to isolate Factor 3 from natural sources using conventional fractionation methods and also techniques specifically developed for this purpose. After identification of selenium as the essential part of the α-Factor 3 molecule these efforts were halted in view of the low yields and the chemical lability of highly purified Factor 3 fractions. Since then, a two-pronged attack on the problem of the chemical structure of Factor 3 has been developed:

a) Screening of organoselenium compounds for biopotency and systematic synthesis of "tailor-made" selenium derivatives are deployed to arrive at synthetic Factor 3-active substances. This approach is carried out in close and enjoyable cooperation with Professor Dr. Arne Fredga of the Institute of Chemistry, University of Uppsala, Sweden, whose superior knowledge and experience in the field of organoselenium chemistry has greatly contributed to the progress reported here. Some of our joint results are presented below.

b) With radioactive selenite-Se^{75}, the pathways of selenium metabolism are being investigated in yeast and in the rat. Dr. Trygve Tuve, working in our laboratory, has devised highly sensitive methods of identification of natural organoselenium compounds employing multiple-system paper chromatography and radioautography. Several unknown such compounds have been detected. By using the labeled unknowns as markers their chemical characterization is being worked out. The work is not further discussed here since it has been dealt with elsewhere during the meeting (25). Suffice it to say that preliminary results indicate that selenium does not necessarily follow the pathways of sulfur metabolism, especially in the rat when it is applied at physiological levels.

In the end it is hoped to combine the outcome of these two approaches by showing that one or the other Factor 3-active, synthetic compound is identical with the naturally occurring active substances.

Factor 3. The research which eventually led to the detection of the role of selenium as an essential dietary agent began about 20 yr ago. It was then discovered (17) that rats maintained on certain vitamin E-free rations low in sulfur amino acids died from a severe necrotic degeneration of the liver (Fig. 1). In earlier studies supplements of two dietary constituents, namely L-cystine (26) and vitamin E (18) had been found to protect against the deficiency. That a further factor, other than L-cystine and vitamin E, was involved became apparent even before tocopherol was found to be protective, but conclusive evidence for the existence of this agent was obtained only after 1949. The agent was not identical to any previously known, chemically identified dietary principle. It was discovered to be present in brewer's yeast (15), so-called vitamin-free casein (16), kidney, liver, and numerous other natural source materials (Schwarz et al., unpublished data). For this principle the term Factor 3 was introduced in 1950, to indicate that it constituted the third natural agent known to affect the development of dietary liver necrosis.

Factor 3 deficiency diseases. As could be surmised on general pathogenetic principles, liver necrosis, though the most important lesion seen in the rat, constitutes only a specialized case of a disease which involves other organs as well. In different species very different disease entities ensue. The mouse reacts to the same dietary conditions primarily with a pronounced degeneration of the heart muscle (Fig. 2), accompanied by peripheral muscle degeneration, liver necrosis, kidney necrosis, and pancreatic dystrophy (4). In birds, such as chicks and turkeys, simultaneous lack of vitamin E and Factor 3-selenium produces exudative diathesis. This entails subcutaneous edema, changes of the serum protein levels, and also lack of growth (19). Especially since the discovery of selenium as the integral part of the Factor 3 molecule has it become evident that the factor protects against a large variety of deficiency syndromes in numer-

[1] The terms selenium and Factor 3 are not synonymous within the logical context of this symposium. Factor 3 designates a principle which in all its properties satisfies the criteria of a dietary agent of wide spread biological significance. Elementary selenium and many organic selenium compounds are not biologically active, while others are highly potent. Until final clarification of the structure of Factor 3, the term Factor 3-selenium is applied to designate Factor 3-active selenium compounds in general.

ous species. A list of deficiency symptoms responsive to Factor 3-selenium is presented in Table 1. The dose levels needed for their prevention, and also cure, are physiological, i.e., they are equal or similar to Factor 3-selenium contents found normally in various dietary ingredients, feeds, or in tissues. Most of the diseases are fatal, and some are economically important. They will be more aptly described in the following articles. Knowledge in this area is still developing rapidly, and other selenium-responsive diseases may be found in the future.

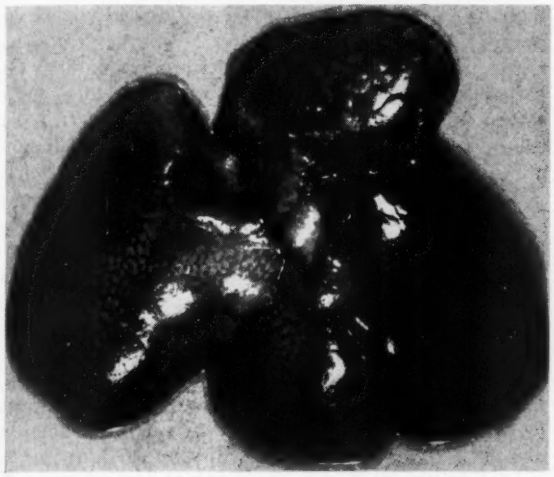

FIG. 1. Dietary necrotic liver degeneration in the rat (14).

FIG. 2. Multiple necrotic degeneration in the mouse (4).

TABLE 1. *Factor 3-Selenium Responsive Deficiency Symptoms in Different Species*

	Growth Deficit	Necrosis			Muscular Dystrophy	Calcifications	Lung Hemor.	Pancreas Atrophy	Serum Protein	Exudative Diathesis
		Liver	Kidney	Heart						
Rat	(+)	+	+	(−)	(+)	+	(+)	(+)	+	−
Mouse	(+)	+	+	+	+		(−)	+	+	−
Rabbit		+					+			
Mink				+	+					
Hog		+			+					
Sheep	(+)			+	+	+				
Cattle				+	+	+				
Chicken	+				(+)				+	+
Turkey	+				(+)				+	+
Horse					+					
Trout	?	?								

+ Pronounced pathological changes; (+) not always detectable; (−) occasionally found; − no pathological changes; ? possible, but not clearly documented.

Interrelation with other factors. In many, but not all of these diseases, the remarkable synergistic correlation of Factor 3 to vitamin E can be observed. The nature of the interrelationship between these two primary protective agents, and also of sulfur amino acids, has been a puzzle into which only recent studies have brought some light. Briefly stated, the results presently available are as follows: L-cystine, originally believed to play a primary role in the protection against liver necrosis, has been shown to contain a trace contamination of ca. 1–2 μg of selenium, which accounts in large part for its biopotency (24). Selenium-free sulfur amino acids in general, however, have a delaying effect on the disease. This is caused by a specific sparing effect on the vitamin E-requirement (20). The tocopherol sparing action of sulfur amino acids can be interpreted in metabolic terms. A specific breakdown of respiratory systems in the liver, respiratory decline, is prevented in vitro by tocopherol or a tocopherol metabolite. Respiratory decline, in turn, is due to the breakdown of enzymes containing sulfhydryl groups, probably more specifically vicinal dithiol groups. The active form of tocopherol appears to relate metabolically to these enzyme sites (2, 13). Dietary sulfur amino acid supplementation keeps the content of such active sulfhydryl compounds, for example of coenzyme A (1) and lipoic acid (Schwarz et al., unpublished data) at adequate levels. Under our standard dietary conditions, the latter are reduced to ca. 60% of normal. At which site Factor 3-selenium exerts its metabolic role is not yet known. Several possibilities, such as specific steps in oxidation-reduction reactions, sulfur-amino acid metabolism, and also protein synthesis are under study.

TABLE 2. *Composition of Basal Diet*

Torula yeast*	30
Sucrose	59
Lard, vitamin E-free†	5
Salts‡	5
Vitamin powder§	1

* Lake State Yeast Corporation, Rinelander, Wis. † Vitamin E-free animal fat, stripped by molecular distillation (Distillation Products Division, Rochester, N. Y.). ‡ $CaCO_3$, 543.0 g; $MgCO_3$, 25.0 g; $MgSO_4$, 16.0 g; NaCl, 69.0 g; KCl, 112.0 g; KH_2PO_4, 212.0 g; $FePO_4 \cdot 4H_2O$, 20.5 g; KI, 0.08 g; $MnSO_4$, 0.35 g; NaF, 1.0 g; $Al_2(SO_4)_3 \cdot K_2SO_4$, 0.17 g; $CuSO_4$, 0.9 g. § Lactose, 88.68 g; thiamine HCl, 40 mg; riboflavin, 25 mg; pridoxine HCl, 20 mg; D-calcium panthothenate, 200 mg; choline chloride, 10 g; niacin, 1 g; menadione (2-methyl-1, 4-naphthoquinone) 10 mg; folic acid, 20 mg; biotin, 10 mg; and vitamin B_{12}, 1 mg. Vitamin A acetate (1 mg %) and vitamin D (0.01 mg %) were added to the diets dissolved in a small amount of ethanol (0.5 cc/100 g of diet).

All available evidence demonstrates that it is fallacious to assume that vitamin E and Factor 3 substitute for each other. They are two different and separate entities. For instance, there are several well known vitamin E deficiencies which are completely unaffected by Factor 3 even in large excess. Resorption sterility in the rat (7), muscular dystrophy in the rabbit (8), and encephalomalacia in the chick (3) are in this group. On the other hand, there are Factor 3 deficiencies which are unaffected by tocopherol, but they are less thoroughly investigated. In our experience, Factor 3-selenium deficiency produces adrenal atrophy, muscular wasting, severe pancreatic dystrophy and diminished growth in rats. However, it is very difficult to produce a diet completely free of selenium. Even certain crystalline amino acids contain appreciable levels of the element.

Factor 3 assay. For the intensive efforts between 1950 and 1957 to isolate Factor 3 from various source materials, prevention of liver necrosis in the rat was used as an assay system. The composition of the basal diet, in use without major variation since 1949, is shown in Table 2. The basic scheme of this ration is not different from other so-called semipurified dietaries, except that 30% Torula yeast is used to supply the protein (14). The diet is suboptimal with respect to sulfur amino acids, and is free of vitamin E. The other vitamins and trace factors, as far as known, are supplied in ample amounts.[2] The protein content amounts to circa 13–15%. The response of our animals to the basal diet is rather uniform. Under well controlled environmental and dietary conditions the inbred Fisher 344 strain of rats succumbs to liver necrosis on the average after 21 days. As a matter of routine, animals are predepleted on the diet until the 13th day. At that time they are allotted to groups of ten and supplemented with test materials which are usually mixed into the ration. For exact comparisons, supplements to be tested are assayed at different dose levels. Usually, tests are terminated on the 30th day, i.e., 17 days after the beginning of supplementation. Results are evaluated by comparison of the average velocities with which liver necrosis develops in various groups. In other words, the reciprocals of the individual survival times are averaged. An "infinite" survival time is assigned to survivors since their reciprocal survival time (= velocity of developing the deficiency) limits towards 0. With graded dose levels of liver protecting agents, approximately straight-line dose response curves are obtained over a wide range, i.e., from 20 to 80% protection. Results are expressed in units, a unit being the amount of Factor 3 necessary in 100 g of diet to afford 50% protection (16).

Natural distribution. The Torula yeast is produced commercially in large scale as a feed ingredient, for instance as a byproduct of cellulose and paper manufacturing. It is grown on spent sulfite waste liquor and is not only free from tocopherol, like other yeasts, but also from Factor 3 potency. Recent determinations showed that it contains on the average about .3 μg of selenium per gram of dry weight, but the element is evidently biologically unavailable. This stands sharply in contrast to brewer's yeast which is a good source of Factor 3 and affords protection when supplemented at 3–5% levels to the 30% Torula diet (15). Only true brewer's yeast obtained from fermentation of wort has this effect, while the so-called "primary grown" *Saccharomyces* yeasts are—like Torula—low or free from Factor 3 activity (12). In the earlier stages of our work on Factor 3, the disparity between Torula yeast and brewer's yeast has been one of the most valid points providing proof for the existence of the then unknown agent. The main reasons for the differences between yeasts appear to lie in the method of cultivation (medium, amount of aeration) and not in a difference between strains of yeast. It seems as if Factor 3 active selenium is present only in those yeasts which are grown or maintained in relative anaerobiosis. In yeasts, and possibly also other living systems, selenium could conceivably be shifted back and forth from an inactive storage form to a Factor 3-active metabolic derivatives, according to the needs of the situation.

Over the years a considerable body of information has been accumulated on the natural distribution of Factor 3. Most materials screened for potency were thoroughly fat extracted to eliminate possible interference by vitamin E. Factor 3 is widely distributed in nature, it is found in materials of microbial, plant and especially animal origin. However, there are wide differences in Factor 3 content, for instance between various organs. The most potent source of Factor 3 detected in these studies was dried, fat extracted sheep kidney powder, 0.2% of which sufficed in the diet to protect fully against death from liver necrosis (Schwarz et al., unpublished data). Even in this material the absolute amounts of Factor-3 selenium are minute: selenium contents range from 10 to 20 μg/g, but comparison of selenium analysis and Factor 3 potency shows that even in kidney not all the selenium is present in biologically active form. The chemical nature of the inactive form

[2] With the exception of the glucose tolerance factor (GTF), identified as trivalent chronium (23).

of selenium in biological materials needs to be investigated further.

Fractionation. For the purification procedure, large amounts of starting material had to be worked up. All in all, several tons of brewer's yeast, kidney, and also casein-digests were used over the years. Methods for the liberation of Factor 3 were worked out for a variety of starting materials.

Like many other essential micronutrients, Factor 3 is contained in natural material in strongly bound form. It seems to be associated with protein. Enzymatic autolysis or acid hydrolysis turned out to be indispensable for its extraction. The concentration of Factor 3 was tedious and time consuming. Its physicochemical properties do not lend themselves easily to development or application of those techniques which are standard for purification of organic essential factors. Problems arose especially in the transfer of laboratory experiments into pilot plant scale, and later on in the elaboration of methods for the final steps of the isolation procedure. Following attempts with enzymatic digests of brewer's yeasts and casein as source materials, a satisfactory pilot plant method was eventually worked out with pork kidney powder.[3] Sulfuric acid hydrolysis was used to liberate the active principle. In the over-all project, more than 4,000 fractions were prepared in the laboratory and evaluated in the animal assay for Factor 3 potency.

Separation of α- and β-Factor 3. It was recognized in 1953 that, at least in kidney, two different components with Factor 3 activity occur. These were designated α- and β-Factor 3. They can be separated by a variety of procedures (Schwarz et al., unpublished data). As far as is known, each behaves subsequently as a distinct chemical entity. In the crude fractionation scheme used for pork kidney powder, the α-Factor 3 separated out in the barium sulfate cake when sulfuric acid was precipitated by barium acetate, after 6–8 hr of hydrolysis (Table 3). α-Factor 3 is adsorbed in this precipitate, either on the barium sulfate itself, or more likely on certain fractions of humic acid which settles with the former in large amounts. Humic acids are well known to have the properties of ion exchangers. β-Factor 3, on the other hand, is contained in the filtrate. From the humic acid/barium sulfate cake α-Factor 3 is eluted in ca. 25-fold concentrated form by a fractionated treatment with alkali, barium hydroxide and ethanol. β-Factor 3 differs from α-Factor 3 in a variety of properties. It is, for instance, extractable from water by phenol while α-Factor 3 stays behind. The two factors are easily and routinely separated in this manner which may indicate that β-Factor 3, in contrast to α-Factor 3, has some cationic properties.

α-Factor 3. After separation of these two fractions with Factor 3 potency, the isolation attempts were concentrated mainly on the α-Factor 3 fraction. Through elaboration of a series of 12–15 reproducible subsequent steps of purification (Foltz and Schwarz, unpublished data), highly active α-Factor 3 preparations were obtained in milligram quantities by routine operation. These fractions can now be calculated to have contained a few percent of Factor 3, the remaining impurities consisting mostly of organic, low molecular carboxylic acids. Numerous attempts to purify this mixture further, for instance, by specific precipitations, chromatography on ion exchanger, silica or cellulose columns, high-vacuum distillation, solvent fractionation, and countercurrent distribution failed, apparently due to the reactivity of the α-Factor 3 molecule. In contrast to many other selenium compounds, α-Factor 3 could not be sublimed, the activity being fully retained in the residue. By most other methods, especially column operations, the activity was irretrievably lost. Other procedures apparently liberated selenite-selenium.

TABLE 3. *Separation of α- and β-Factor 3*

Kidney Powder
(Defatted, Dried)
H_2SO_4-Hydrolysis
Ba-Acetate Neutralization

$BaSO_4$-humic acid cake	Filtrate and wash
Alkaline ethanol-$Ba(OH)_2$-fractionation	
α-Factor 3	*β-Factor 3*
not phenol extractable	phenol-extractable

Selenium. The presence of selenium, at first ascertained by spot tests using the methylene blue-sodium sulfide method (5), was discovered by virtue of the fact that highly active Factor 3 fractions developed a characteristic, garlic-like odor when made alkaline. A series of analyses of Factor 3 concentrates of varying origins and very different degrees of purification showed a correlation between selenium contents and potency in the bio-assay against liver necrosis in the rat (21). Most important, it was found immediately that known organic as well as inorganic selenium compounds were highly potent in affording protection against liver necrosis. However, different selenium compounds showed widely different activities; some were completely inactive. In spite of this degree of chemical specificity in the Factor 3 activity of selenium compounds, it is most unlikely that the factor itself is of vitamin nature. Comparison of injected versus fed doses of selenite-selenium showed no difference in activity. Similarly, the feeding of sulfanilamides, while largely suppressing the gastrointestinal flora, did not change the biopotency of selenite (Schwarz et al., unpublished data). While these experiments would tend to show that assimilation of selenite to the organically bound forms of Factor 3 occurs within the tissues of the rat organism, conclusive evidence on this

[3] Much of the pilot plant work has been carried out by Schwarz Laboratories, Inc., Mt. Vernon, N. Y. The most efficient and knowledgeable cooperation of the members of their staff, particularly of David R. Schwarz and Lionel C. Reed, is gratefully acknowledged.

TABLE 4. *Biopotency of α-Factor 3*

	ED_{50}, μg % Se
1. 3/5016, Purified α-Factor 3 concentrate from pork kidney	.72 (*Radio activation analysis*)
2. 3/5329, Purified α-Factor 3 concentrate from pork kidney	.69 (*Radio activation analysis*)
3. Average from H_2SO_4 digests of 3 different, purified α-Factor 3 fractions* (22).	.73 (*Codeine method*)

point would be gained only by germfree-animal experimentation.

Biopotency of selenium compounds. For the comparison of biopotencies of various compounds the 50% effective dose levels (ED_{50}) were established by serial bio-assays at varying levels of supplementation (22). The results were related to the selenium content, i.e., each ED_{50} is expressed in micrograms of selenium supplemented per hundred gram of diet (μg % selenium) to afford 50% protection. Determinations of selenium by various methods in our purified preparations of known biopotency produced a figure of approximately .7 μg % selenium for the ED_{50} of Factor 3 (Table 4). This constitutes a maximal value. Since the animals eat ca.6 g of diet per day, a daily dose of .04 μg of Factor 3 selenium protects half, while .1 μg does so completely over the time of our assay.

Factor 3 selenium is at least three times as potent as inorganic selenium compounds. Elementary selenium is inactive. Of most inorganic selenium compounds, for instance, selenite or selenate approximately 2–3 μg % selenium are required for 50% protection over the experimental period of 30 days.[4] For exact comparison of biopotencies, selenite can be used conveniently as a reference standard.

Effective dose levels of various selenium compounds taken rather at random from a total of ca.200 substances assayed since 1957 are shown in Table 5. As summarized previously (22), three main categories can be differentiated: a large group is fully inactive or needs to be supplied at high dose levels in order to produce protection, being as ineffective as elementary selenium or nearly so. Biological inactivity can naturally be due to a number of reasons: the material may not be absorbed from the gut; it may not be broken down effectively to make the selenium available; it may be instable and precipitate elementary selenium; it may be excreted so effectively from the organism that it cannot be used; and, lastly, it may be converted more rapidly into the hypothetical, inactive form of organoselenium than into the active one. For several of these mechanisms examples are on hand.

A second, relatively large group of compounds shows an ED_{50} of 2–3 μg % selenium, i.e., these substances will protect when added at levels equivalent to those required of selenite- or selenate-selenium. This might indicate that the organism is efficient in breakdown and metabolism of a wide variety of organoselenium derivatives. They all may be utilized for the production of Factor 3 within the organism, i.e., the selenium atom may be liberated and used, like selenite selenium, as a building stone of the selenium derivative which is ultimately effective in metabolism. The selenium analogues of the sulfur amino acids, specifically cystine, cystathionine, and methionine, are in the second category. They can be utilized by the rat for Factor 3 activity, but they are not more potent than selenite. It follows from this observation that these substances are not identical with α-Factor 3; they also do not show the chemical properties which are characteristic of the latter.

The third group contains substances which are—like Factor 3—more effective than selenite. Thus far, only a very few such compounds are known, and none of them surpasses the ED_{50} of .7 μg % selenium characteristic of α-Factor 3. We like to assume that some of these compounds are closely related to Factor 3. However, since the metabolically active form of selenium in the organism is presently unknown, it remains to be established how closely α-Factor 3 relates to it. Other hypothetical reasons for enhanced potency, such as more quantitative absorption or more efficient handling in the interconversion to Factor 3 must be taken into consideration.

On the whole, it must also be understood that these figures for biopotency are valid only for the rat. From published results a few comparisons can be made between rat and chick assays carried out with identical preparations (19). There is good agreement of the relative potencies in both tests. Yet, there can be no doubt that in other species, possibly most prominently in ruminants, the relative potencies of various selenium compounds might be different.

Synthetic approaches. From the behavior of α-Factor 3 in the course of the fractionation procedures, from systematic stability tests at various phases of purification, and from other chemical and physical investigations with Factor 3 preparations certain concepts of the chemical nature of α-Factor 3 have evolved. For example, the α-Factor 3 molecule has strongly acidic properties, it contains selenium most likely in the oxidation state of selenite (+4), or possibly selenate (+6), it is of low

TABLE 5. *Relative Potencies of Selenium Compounds Against Dietary Necrotic Liver Degeneration*

	ED_{50},* μg % Se
Factor 3	.7
Sodium selenite	2.2
Selenocystine	2.4
Benzeneseleninic acid	6.7
Selenouracil	ca. 60
Phenylselenide	134
Selenium (gray modification)	Inactive (>300)
4-Carboxybenzeneseleninic acid	Inactive (>300)

* Effective dose level required for 50% protection.

[4] Long term studies have shown that 10 μg % selenite-selenium will protect the animals over their entire life span, in the absence of vitamin E. Under these conditions, a severe tocopherol deficiency disease develops which afflicts mainly the central nervous system (10).

molecular weight, and it is nonaromatic. All available observations point to the possibility that α-Factor 3 is an aliphatic seleninic or selenonic acid. It is on this premise that the collaborative studies with Dr. Arne Fredga and his colleagues are based.

Assays of mono-selenium di-carboxylic acids of the type $Se((CH_2)_nCOOH)_2$ produced evidence that different members of this series are utilized to greatly different extents (Table 6). While the acetic, propionic and butyric acid derivatives are rather inactive, the seleno-δ,δ'-di-valeric acid is quite effective. However, it does not surpass selenite.

The inactivity of the three lower mono-selenium derivatives and many other similar compounds tested is contrasted by the fact that the corresponding di-seleno derivatives of carboxylic acids are utilized quite well (Table 7). In this series of compounds, biopotency increases with the distance of the selenium from the carboxyl group. An optimum of biopotency is found when the element is in the γ-position from the carboxyl group. The butyric acid derivative is slightly, but distinctly more potent than selenite.

It has been known since before 1935 that the aliphatic di-seleno-di-acids are very readily oxidized in vitro to the corresponding seleninic acids (6), especially under neutral or slightly alkaline conditions:

$$COOH\ (CH_2)_nSe\ Se\ (CH_2)_nCOOH + 3\ O_2/_2 + H_2O \rightleftharpoons 2\ SeO_2H\ (CH_2)_nCOOH$$

This oxidation is easily reversible. The reaction is quite likely to occur in vivo as well. Animal assays with the seleninic acids, obtained by oxidation of the di-seleno-di-acids, gave results indistinguishable from those with the reduced forms. In view or this interchangeability, and since the di-seleno compounds are handled more conveniently, most subsequent studies were carried out with the di-seleno forms. Tests with all possible isomers of di-seleno-di-butyric acid and with similar series of compounds showed that enhanced biopotency is encountered only with substances which have a normal, straight chain of carbon atoms.

TABLE 6. *Monoseleno-di-n-carboxylic Acids*

	ED_{10}, μg % Se
Seleno-diacetic acid $Se(CH_2—COOH)_2$	22
Seleno-β,β'-dipropionic acid $Se(CH_2—CH_2—COOH)_2$	55
Seleno-γ,γ'-dibutyric acid $Se(CH_2—CH_2—CH_2—COOH)_2$	63
Seleno-δ,δ'-divaleric acid $Se(CH_2—CH_2—CH_2—CH_2—COOH)_2$	2.5

TABLE 7. *Biopotency of Di-seleno-di-carboxylic Acids*

$$\begin{array}{l} Se\text{-}(CH_2)n\text{-}COOH \\ | \\ Se\text{-}(CH_2)n\text{-}COOH \end{array}$$

	ED_{10}, μg % Se
Di-seleno-di-n-acetic acid	2.3
Di-seleno-di-n-propionic acid	1.9
Di-seleno-di-n-butyric acid	1.8
Di-seleno-di-n-valeric acid	2.6

TABLE 8. *Di-seleno-di-n-carboxylic Acids Various Positions of Selenium on Carbon Chain*

	ED_{10}, μg % Se
Di-seleno-α,α'-di-n-butyric acid $(CH_3—CH_2—CH(Se)—COOH)_2$	3.4
Di-seleno-β,β'-di-n-butyric acid $(CH_3—CH(Se)—CH_2—COOH)_2$	3.1
Di-seleno-γ,γ'-di-n-butyric acid $(CH_2(Se)—CH_2—CH_2—COOH)_2$	1.8
Di-seleno-γ,γ'-di-n-valeric acid $(CH_3—CH_2—CH(Se)—CH_2—COOH)_2$	1.4
Di-seleno-δ,δ'-di-n-valeric acid $(CH_2(Se)—CH_2—CH_2—CH_2—COOH)_2$	2.6

Biopotency enhanced further over that of the γ-substituted butyric acid was obtained with γ,γ'-di-seleno-di-valeric acid (Table 8). This synthetic compound is a racemic mixture. It shows an ED_{50} of 1.4 μg % selenium, i.e., it is half as potent as α-Factor 3. Experiments to clarify whether one of the stereoisomers is similar in biopotency to Factor 3 are under way. However, the deracemization of the compound is difficult; thus far it has been achieved only in part. Alternate synthetic approaches to the stereoisomers of γ-seleno-valeric acid, or of derivatives thereof with biological activity, have been developed more recently by Dr. Fredga and his collaborators.

Several other aspects of the interrelationship of structure and potency of selenium compounds are under investigation by the same approach. At any rate, from the examples presented here, it is clear that synthetic organoselenium compounds with properties similar to those of α-Factor 3 and with biopotencies surpassing that of selenite-selenium can be obtained. The γ,γ-di-seleno-di-valeric acid is currently being used for clinical trials.

Methods of selenium determination. Even though selenium determination cannot substitute for assays of Factor 3 activity, a reliable procedure of analysis of tissues, nutrients, and other specimens of biological origin would be most desirable for physiological, nutritional, and especially also clinical studies.[5] Several colorimetric

[5] In several countries, laws require specifically that foods contain "no" selenium. These laws were made before recognition of the fact that almost all important foods and feeds contain small, but appreciable amounts of the element, at a time when methods for determination of these quantities of selenium were not available.

or titrimetric methods are available, the sensitivity of which would at least in theory suffice to assay the small amounts of the element which elicit the pronounced protective effects discussed here. In our group we have given careful study to many of these techniques. In spite of their sensitivity almost all existing procedures have turned out to be inadequate for the purpose. There are several reasons: the most important difficulty in the assay, and one which has been overlooked in many of the older attempts to find the element in biological specimens, is a severe and frequently complete loss of selenium during the digestion procedures routinely applied. Chloride and other halogens are present in most samples of biological nature. Selenium, usually amounting to 0.1–5 μg/g of dry matter, seems to react with these elements and disappears from dry and wet ashing mixtures as a halogen derivative. The complication could be overcome by the development of digestion methods in closed systems. These, however, such as the sodium peroxide bomb or the Schöniger combustion method (11), do not have the capacity for the amounts of specimen needed. In dry ashing methods, sublimation of SeO_2 is a further cause of loss. Another, but less serious reason for errors is the loss of selenium, mostly as SeO_2, during the drying of specimens and during dry storage. Such losses happen even in the course of lyophylization, as can be ascertained with radioactive biological materials.

Provided that these difficulties can be overcome, there still remains the necessity to study carefully the adequacy of the method for each *type* of sample to be analyzed. As a standard for recovery studies, selenite, which can be determined easily, should not be used; organoselenium compounds must be applied.

At present radioactivation analysis (9) seems to be the method of choice. In analyzing for selenium, the sample in its crude, liquid or dry form is irradiated in a nuclear reactor or by another source of neutrons. Through an n,γ-reaction, one of the natural isotopes of selenium is converted into radioactive Se^{75}, which has a half life of 128 days and is easily handled. Subsequently, a carrier amount of unlabeled selenium is added in large excess (20 mg) before digestion of the material, and care is taken that wet ashing is thorough and complete. Following distillation with bromide, selenium is isolated in elementary form and counted for radioactivity. The losses occurring during digestion would be critical if only a very small quantity of selenium were present. Due to the carrier-selenium they become irrelevant. It must be emphasized that success even of this method depends on careful execution and on the elaboration of suitable controls and techniques for each type of sample. More recently, efforts have been successful to determine the element by a nondestructive technique, i.e., by direct measurement of the activated selenium in the specimen by means of a sensitive γ-spectrometer coupled with a multichannel analyzer.

Clinical outlook. The results in animals under experimental as well as field conditions could be taken as indication that Factor 3-active selenium compounds might have useful applications in the human. We have quite intentionally refrained from such applications in order to satisfy several preliminary requirements, but are now in a position to proceed. One of the prerequisites of therapeutical applicability is an adequate margin of safety. The ratio between the daily, fully protective dose of selenite-selenium fed in the diet, on one hand, and the minimum 100% lethal dose when injected intraperitoneally, on the other hand, has been established in animals of the same strain and age under identical dietary and environmental conditions. It was found to be approximately 1:3,000. Thus, even for selenite-selenium, the therapeutic margin is unusually large; it compares very favorably with that of almost all other dietary factors. For active organoselenium compounds this ratio is still larger, since these substances are less toxic and show greater biopotency. For γ,γ′-di-seleno-di-valeric acid, a ratio of at least 1:12,000 has been obtained (Schwarz et al., unpublished data).

The first clinical application of this compound has been attempted in children suffering from kwashiorkor. For a number of reasons we have been under the impression that the commonly accepted interpretation of kwashiorkor as a "protein malnutrition" disease does not fully explain the disease picture. There were several findings indicating the possibility that Factor 3, bound to and normally associated with protein, may play a role. Preliminary trials were carried out by Drs. John Waterlow and Robert Montgomery at the Tropical Metabolism Research Unit, Kingston, Jamaica. They used a type of case which is relatively infrequent and which is characterized by very slow weight gains after overcoming the initial, acute phase. Two children who had not gained weight for 2 and 5 weeks, respectively, were given daily supplements of 25 μg of selenium in form of γ,γ′-di-seleno-di-valeric acid. They responded immediately with gains of 450 and 660 g over 10–14 days of treatment; this was accompanied by an increase in food intake. The result is presented merely to indicate the direction in which we hope to expand our further efforts. The finding is very preliminary indeed, but it suggests that eventually we may have to add the human to the list of species in Table 1 which show Factor 3-selenium responsive diseases.

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Nutritional effects of selenium compounds in chicks and turkeys

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SELENIUM IS OF IMPORTANCE in the nutrition of chickens and turkeys because of the relationship of selenium to certain diseases previously ascribed to a deficiency of vitamin E. With proper dietary conditions, three distinct deficiency diseases can be produced in young chickens that respond to vitamin E supplementation. These are called exudative diathesis, encephalomalacia and muscular dystrophy. Each of these can be produced, uncomplicated by the others, depending on dietary conditions used. Other vitamin E deficiency diseases can be produced in adult birds which are still different from the ones described in the young animal. None of the above conditions is an uncomplicated vitamin E deficiency since many compounds other than vitamin E affect the course of these diseases. The discovery that dietary selenium also will prevent some of these deficiency diseases has led to extensive investigations of the nutritional role of selenium in birds. It is the purpose of this review to summarize recent findings on nutritional effects of selenium in chickens and turkeys.

Effect of Selenium on Exudative Diathesis

The nutritional disease in chicks and poults most sensitive to dietary selenium supplementation is exudative diathesis. The chicks shown in Figure 1 illustrate the gross appearance of the disease. This condition, first described by Dam and Glavind (9), is characterized by an accumulation of fluid throughout the body with greatest amounts found under the skin on the breast and abdomen. Small hemorrhages are commonly found in tissues adjacent to large accumulations of fluid. The plasma proteins of chicks with exudative diathesis are lowered in concentration and the albumen fraction appears to be particularly depleted (17). The exudate fluid is usually of bluish color, and similar in composition to blood plasma. The proteins of the exudate fluid have an electrophoretic mobility similar to the plasma proteins of the affected chicks. Changes in permeability of the capillary wall to blood proteins may be the cause of the widespread edema although the possibility of less plasma protein synthesis has been suggested (7, 17). Capillaries in chicks with exudative diathesis are more permeable to trypan blue dye than in chicks not having the disease (10).

An anemia has been described in chicks with exudative diathesis that has been characterized both as microcytic (39) and macrocytic (30). The anemia is probably related to the hemorrhages that occur. Dietary factors other than vitamin E known to affect exudative diathesis prior to the discovery of the effect of selenium have been reviewed by Ames (2).

Exudative diathesis can be produced by feeding several types of diets to young chicks, but the most severe cases of the disorder are produced on diets containing a high level of Torula yeast (39). These are modifications of the diet which Schwarz (32) reported to cause liver necrosis in rats. Probably chicks fed diets containing Torula yeast readily develop exudative diathesis because (*a*) these diets are low in selenium or at least in available selenium (35), (*b*) the yeast is high in ash and unsaturated fatty acids (5), and (*c*) the yeast may contain a selenium or vitamin E antagonist (29), perhaps in the form of pro-oxidants.

In studies by Scott et al. (39), a large number of antioxidants were found to be ineffective against exudative diathesis in chicks fed diets containing Torula yeast. Several antioxidants had been previously reported to be effective against exudative diathesis when diets did not contain Torula yeast (11). However, recently a level of ethoxyquin higher than used by these workers (0.10%) was reported by Machlin and Gordon (20) to be effective against exudative diathesis in chicks fed diets containing Torula yeast.

The effectiveness of selenium against exudative diathesis in chicks was reported simultaneously by Patterson et al. (28) and Schwarz et al. (34). Selenium, as sodium selenite or in an organic selenium compound, seleno-cystathionine, was found to be effective in preventing exudative diathesis when included in diets at very low levels. Patterson et al. (28) found that dietary selenium levels of 0.3 mg/kg were completely effective against the disease, and Schwarz et al. (34) reported that 0.1 mg/kg was sufficient. Elemental selenium was much less effective against the disease.

Subsequent to the original studies cited above, the

observation that selenium will prevent exudative diathesis has been confirmed by many investigators (12, 24, 29, 31), who used diets with several protein sources including Torula yeast. All of the manifestations of exudative diathesis, including low plasma proteins and anemia, are corrected by the addition of selenium to the diet (24, 31).

The data shown in Table 1 illustrate the effectiveness of very low dietary selenium levels against exudative diathesis. In the study shown here, 0.08 mg Se/kg of diet as sodium selenite completely prevented the disease and lower levels markedly reduced the incidence. Minimum added selenium levels required to prevent exudative diathesis apparently range from 0.05 to 0.1 mg/kg of diet, depending on the type of diet used (4, 24, 30).

Selenium is probably not stored in a biologically active form for very long periods of time. Sondergaard et al. (40) showed that chicks which received Torula yeast diets containing 1 mg/kg of selenium for the first week of life showed symptoms of exudative diathesis approximately one week later than others that received the unsupplemented Torula yeast diet from hatching.

FIG. 1. Two chicks with exudative diathesis. *Left:* Note subcutaneous exudates and discoloration. *Right:* Note hemorrhages in lower abdominal wall, and older hemorrhage in subcutaneous tissue of the neck.

TABLE 1. *Levels of Selenium Effective Against Exudative Diathesis**

Treatment	Exudative Diathesis
Basal†	16/16‡
+ 0.02 mg selenium/kg	14/16
+ 0.04 mg selenium/kg	5/15
+ 0.06 mg selenium/kg	1/17
+ 0.08 mg selenium/kg	0/16
+ 0.10 mg selenium/kg	0/16

* From Nesheim and Scott (24). † Basal diet contains 58.5% Torula yeast; selenium levels supplied by Na_2SeO_3. ‡ Expressed as number of chicks showing symptoms (numerator) over number of chicks surviving (denominator) until symptoms appeared.

TABLE 2. *Effect of Selenium on Chick Growth in Presence of Adequate Vitamin E**

Treatment†	Average Weight, 5 Weeks, gm
Basal (160 mg/kg d-α-tocopheryl acetate)	450(32)‡
+ 0.04 mg selenium/kg	507(32)
+ 1.0 mg selenium/kg	522(32)

* From Nesheim and Scott (24). † Basal diet contains 58.5% Torula yeast; selenium levels supplied by Na_2SeO_3. ‡ Figures in parentheses represent surviving chicks. Duplicate groups of 16 White Plymouth Rock male chicks started per treatment.

Exudative diathesis can be produced in poults by feeding diets containing Torula yeast although the edema is much less severe than in chicks (6). Dietary selenium will prevent all of the manifestations of exudative diathesis in this species (30). Levels of selenium as low as 0.5 mg/kg were effective against the disease in turkeys.

Effect of Selenium on Growth

The effect of selenium on growth rate of chickens appears to depend on the type of diet fed.

Using diets containing Torula yeast, Nesheim and Scott (24) showed that growth of chicks receiving dietary supplements of selenium or selenium plus vitamin E grew at a significantly greater rate than chicks receiving a high level of vitamin E alone. The data shown in Table 2, illustrate the improvement in growth rate observed from added selenium when diets containing Torula yeast are used. The diet contained a level of vitamin E that was 4 to 5 times greater than amounts usually needed to prevent exudative diathesis. When vitamin E was present, no more than 0.04 mg of selenium per kg of diet was necessary for maximum growth. Zalkin et al. (41) recently published data indicating an improved growth rate of chicks receiving selenium or selenium plus vitamin E as compared to vitamin E alone, although these authors attributed this to an inadequate level of vitamin E used. No indication of the levels of vitamin E or selenium supplementation studied were included in their paper. Studies by Reid et al. (31) indicated that levels of 1 mg of selenium per kg or higher depressed chick growth. Lower levels of selenium were not markedly growth stimulating when chicks were fed diets containing Torula yeast.

When diets other than Torula yeast are used, there have been no reports of growth stimulation from selenium. Nesheim and Scott (25) reported that no improvement in chick growth rate from dietary selenium additions could be obtained using diets containing crystalline amino acids as a nitrogen source. Neutron activation analysis of this diet indicated that it contained

approximately 0.04 mg of selenium per kg. This amount of selenium was found to be sufficient for maximum growth rate when added to Torula yeast diets containing adequate vitamin E.

The dietary requirement for selenium for growth may be so low that the small amount of selenium present in highly purified diets for chicks is sufficient. Torula yeast diets may contain completely unavailable selenium or increase the selenium requirements so that growth effects may be demonstrated. Presently, the question of a separate growth stimulating effect from selenium compared to vitamin E is difficult to resolve because of the low dietary levels that must be produced.

Muscular Dystrophy

Muscular dystrophy in chicks has a very complex dietary etiology. To produce muscular dystrophy, chicks must be fed diets low in vitamin E and sulfur amino acids (13, 22). Addition of either vitamin E, methionine or cystine to the diet will prevent symptoms from occurring. At least under some conditions antioxidants also will prevent muscular dystrophy in chicks (21, 22). A relationship between arginine level in diets containing casein and incidence of muscular dystrophy also exists (26).

The muscular dystrophy of chicks, first described by Dam et al. (13), is characterized by appearance of white striations in the muscle on the breast of chicks and less frequently on the legs. The gross appearance of breast muscles of chicks with muscular dystrophy is shown in Figure 2. Diagnosis is readily made on autopsy by the grossly visible white striations. The "white" muscle of chicks appears to be affected more than the "red" muscle (23). In this respect, the muscular dystrophy in the chick is similar to that found in the rabbit (16). No apparent disability is evident in chicks severely affected. At least under certain conditions, the muscle lesions are spontaneously repaired as the chicks get older (unpublished data, Cornell University).

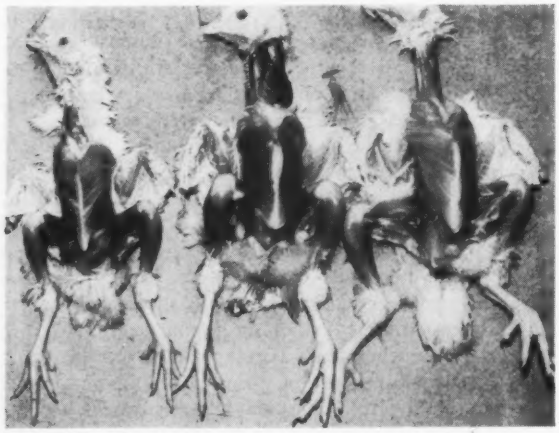

FIG. 2. Chicks on left and right show muscular degeneration as white striations in breast muscle. The chick in the center shows no muscular degeneration and was fed a diet adequate in vitamin E.

TABLE 3. *Effect of Selenium on Muscular Dystrophy**

Treatment†	Muscular Dystrophy
Experiment 1	
Basal	19/20‡
+ 80 mg *d*-α-tocopheryl acetate/kg	0/21
+ 1.0 mg selenium/kg	7/21
Experiment 2	
Basal	13/17
+ 80 mg *d*-α-tocopheryl acetate/kg	0/15
+ 0.1 mg selenium/kg	14/18
+ 5.0 mg selenium/kg	4/13
+ 0.40% DL-methionine	0/17

* From Nesheim and Scott (24). † Selenium levels supplied by Na_2SeO_3. ‡ Expressed as number of chicks showing symptoms (numerator) over number of chicks surviving (denominator) until symptoms appeared.

Histologically, Dam et al. (13) characterized the dystrophic muscle as showing marked degenerative changes with perivascular infiltration with "round" cells. The dystrophy in the chicken resembles the dystrophy of vitamin E deficiency found in guinea pigs and rabbits, in histological appearance.

Dietary selenium has much less effect on muscular dystrophy in chicks than on exudative diathesis. The experiments presented in Table 3 show that comparatively high levels of dietary selenium, 1–5g/ mkg, will reduce the incidence of muscular dystrophy in chicks, but does not completely prevent it. A low level of selenium, 0.1 mg/kg, had no effect on the incidence of dystrophy in experiment 2 shown in the table. Vitamin E and DL-methionine completely prevented the muscular degeneration. The partial effectiveness of comparatively high levels of selenium against muscular dystrophy was also shown by Dam and Sondergaard (14). Levels of selenium of 0.1 to 0.5 mg/kg do not appear to affect the incidence of muscular dystrophy in chicks (4, 24). Apparently, the level of selenium that will prevent exudative diathesis in the chick is quite different from the level of selenium that will have any effect on muscular dystrophy. The relative ineffectiveness of selenium against muscular dystrophy in chicks indicates that the effect of methionine and cystine in this disorder is not due to their selenium contamination. Since levels as low as 0.15% added cystine will prevent muscular dystrophy with certain diets (38), the amount of selenium added by the cystine would be very small compared to levels necessary for affecting muscular dystrophy.

Effect of Dietary Selenium on Other Vitamin E Deficiency Diseases in Birds

In addition to muscular dystrophy and exudative diathesis, several other vitamin E deficiency diseases in chickens and turkeys have been described.

Encephalomalacia, the first recognized vitamin E deficiency disease in chicks (27), is not prevented by

dietary selenium (12). This disease is characterized by a degeneration of the cerebellum of chicks, resulting in a peculiar lack of locomotor coordination that has suggested the name "crazy chick disease" for this syndrome. An extremely large variety of compounds with antioxidant properties can substitute for vitamin E in preventing encephalomalacia.

A lack of vitamin E lowers hatchability of eggs of turkeys and chickens. Creger et al. (8) reported that sodium selenite had no effect on lowered hatchability of turkey eggs when added to diets to supply a selenium level of 0.1 mg/kg. Vitamin E was effective under the same conditions in markedly improving hatchability.

Recently Jensen et al. (18) reported that eggs produced by hens fed diets containing Torula yeast showed low hatchability. Early mortality of chicks hatched from these eggs was high. Vitamin E improved hatchability and chick viability when given to hens fed this diet, but selenium was without effect.

The enlarged hock disorder of turkeys described by Scott (36) does not seem to be affected by selenium (unpublished data, Cornell University). Vitamin E has been reported to be important in this condition (37).

Two other reported vitamin E deficiency disorders of chickens and poults, testicular degeneration in the cock (1) and gizzard myopathy in turkeys (19), have apparently not been studied with respect to selenium. Recently Ferguson and Couch (15) have described degeneration of skeletal muscle in turkeys produced under the same conditions that cause muscular dystrophy in chicks. No information was given as to the effect of selenium on this condition.

Effect of Selenium on Lipid Oxidation

At least one important biological role of vitamin E is that of a potent biological antioxidant. Recently studies have appeared that suggest that vitamin E and selenium have related antioxidant properties.

Bieri (3) reported that *in vitro* lipid oxidation of incubated liver and muscle homogenates of chicks, as measured by formation of thiobarbituric acid reactive products, could be markedly reduced if vitamin E, selenium or cystine was included in diets of chicks from which tissues were obtained. The direct addition of cystine or inorganic selenium to the homogenates did not cause reduced thiobarbituric acid values, suggesting that these materials do not act directly in preventing lipid oxidation.

These studies were confirmed and extended by Zalkin et al. (41) who studied thiobarbituric acid reactive products in liver, heart and leg muscle from chicks fed Torula yeast diets unsupplemented and supplemented with vitamin E or sodium selenite. Chicks fed the basal diets showed evidence of thiobarbituric acid reactive products in tissues on short incubation, whereas these were reduced in chicks supplemented with vitamin E and selenium. The thiobarbituric acid values of tissues following incubation were highest in basal groups and significantly lower in tissues from chicks fed vitamin E or selenium.

Organic selenium and sulfur compounds were found to be effective in reducing the initial rate of hemoglobin catalyzed linoleate oxidation, whereas sodium selenate and selenite were poor antioxidants in this *in vitro* system. As a result of their studies, Zalkin et al. (41) suggest that lipid peroxides form in vitamin E deficient animals, causing tissue damage characteristic of the deficiency, and that dietary selenium compounds lead to the *in vivo* formation of antioxidants capable of reducing lipid peroxidation.

The demonstration of tissue antioxidant activity following ingestion of small amounts of selenium, cystine and vitamin E provides a possible explanation of the similar biological effects of these chemically diverse substances. However, this in itself is not conclusive evidence that this is the manner in which they function to prevent various nutritional deficiency diseases. A specific cause and effect relationship between lipid peroxidation and deficiency diseases observed has not been established. Schwarz (33) has recently discussed the relationship of lipid peroxidation to vitamin E and selenium function. He considers vitamin E and selenium to be independent dietary agents which may act in alternate pathways of metabolism. The relationship between vitamin E and selenium may only be an apparent one because of the specific deficiency diseases that result when simultaneous dietary deficiencies of both are produced. The final assessment of the role of selenium in intermediary metabolism must await future developments in research in this field.

SUMMARY AND CONCLUSIONS

The most marked nutritional effect of dietary selenium in chickens and turkeys is its effectiveness in preventing exudative diathesis. Very low dietary levels are required (0.05–0.1 mg/kg of diet). Selenium is not completely effective against muscular dystrophy in chicks, and levels of selenium that partially prevent muscular dystrophy (1–5 mg/kg of diet) are much higher than the levels effective against exudative diathesis. Selenium appears to have growth stimulating properties for chicks receiving diets containing Torula yeast adequate in vitamin E. Selenium has not been found to be growth stimulating when other types of diets are used, probably since they contain traces of the element.

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A review of selenium responsive diseases of New Zealand livestock

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DURING THE LAST TWO YEARS selenium has become a trace element of paramount importance to the economy of New Zealand. We have found that it will prevent and control several distinct disease entities occurring in all classes of livestock. Some of these entities appear to be closely related pathologically while others appear unrelated.

It is our intention to give an illustrated description of the various disease conditions as they occur in New Zealand and to give details of the work we are carrying out in an endeavor to control them. Some of the conditions mentioned have not yet been shown in controlled experiments to be responsive to selenium, but they have been included in this paper as they have close similarities to those which have responded. Some of the data presented in this paper has not yet been published and is derived from recent selenium field trials (Hartley) and selenium pasture analyses (Grant). The main part of this paper is devoted to the description of field observations and the results of field trials. Thereafter, the histopathology of various selenium responsive conditions is treated briefly, and the results of selenium analyses in tissues and forage are discussed.

DISEASE CONDITIONS ASSOCIATED WITH MYOPATHY

Under this heading the following conditions will be discussed:

Sheep: congenital white muscle disease, delayed white muscle disease, and white muscle disease in hoggets.

Cattle: white muscle disease in calves.

Pigs: hepatosis diaetetica.

Horses: white muscle disease in foals, "tied up" racehorses.

Poultry: exudative diathesis and white muscle disease in chicks, and white muscle disease in pullets.

Sheep

Sheep are kept outside the year round in New Zealand. They are mated in the autumn to lamb in early spring. During winter, in addition to grass they may be break fed on brassica crops and/or supplemented by home grown, usually legume, hay. At lambing the ewes are put on to autumn-saved pasture. Much of the country where the selenium responsive diseases occur was until recent years largely underdeveloped or "run out." However, following cultivation, seeding with improved grasses and clovers, and the liberal application of superphosphate, this type of country now produces lush spring and autumn growth of clovers. If there is excessive rain the pastures grow even more luxuriantly and it is usually under these circumstances that the conditions responsive to selenium can be expected to be most severe.

Field observations. White muscle disease (WMD) in lambs occurs in most areas of the South Island of New Zealand, particularly on the stony and silty soils of the Canterbury Plains. Isolated outbreaks have occurred on the heavier soils, and also on the pumice soils in the center of the North Island. White muscle disease in hoggets (9-month-old sheep) mainly occurs on the heavier soils of the southern part of the South Island. Fig. 1 gives a general picture of the distribution of these myopathies.

CONGENITAL WHITE MUSCLE DISEASE. This entity was first recognized in 1958 when it was responsible for heavy neonatal losses of lambs in parts of the South Island (8). It has also been observed on the pumice soils in the center of the North Island. Affected lambs are either born dead or die within 24 hr of birth. Sudden exertion such as running or suckling often brings on severe respiratory distress followed by death.

Post mortem examination in the majority of cases, shows a marked excess of clear straw colored fluid, often with strands or sheets of fibrin, in all serous cavities, together with an enlarged congested liver. The heart shows mild to most severe subendocardial focal or diffuse grayish white discolorations which extend up to 1 mm into the myocardium. Sometimes the subepicardial myccardium is also involved. These lesions are usually present in one or both ventricles and are seen, in some cases, also in the auricles. Only on rare occasions is the skeletal musculature involved.

(DELAYED) WHITE MUSCLE DISEASE. This condition has been recognized in New Zealand for 10 yr (10, 11) and undoubtedly occurred prior to this. The incidence

TABLE 1. *Effect of Selenium and Vitamin E Therapy on Neonatal Lamb Mortality*

Ewe Treatment	Lamb Deaths Attributable To	
	White muscle disease	Other causes
Selenium	20	20
Vitamin E	54	25
Untreated	41	18

varies greatly from season to season and also between mobs of ewes and lambs on the one property in the one year. The disease apparently develops after birth and is seen in lambs of a few days of age up to 3 months of age; it occurs predominantly between 3 and 6 weeks after birth. The majority of outbreaks are seen at pasture and are apparently unassociated with excessive exercise. On some properties, however, where there have been a few cases in lambs at pasture, driving has precipitated a severe outbreak.

The post mortem findings in this entity have previously been described in detail (10, 11) and differ in no way from those reported in the U.S.A. and elsewhere, as outlined and illustrated in the following paper.[1]

WHITE MUSCLE DISEASE IN HOGGETS. This disease has been known as a clinical entity in the southern half of the South Island for many years: however, it was not recognized as a specific myopathy until 1950 (11). It occurs in 9-to 12-month-old sheep which have been wintered for from 3 to 5 months on turnips and swedes. In some seasons, particularly when the climatic conditions are cold and wet, numerous mobs of sheep develop the disease following driving. The signs of WMD come on quite suddenly, either during or following driving, with rapidly developing listlessness, stiffness, inability to stand, prostration and in the more acute cases death within 24 hr. Less acutely affected animals may be recumbent for 3 or 4 days followed either by death or gradual recovery. Many acutely affected hoggets pass a red-brown colored urine.

The lesions of WMD in many acute cases are not always readily detectable on post mortem examination. Affected musculature is paler than normal with a tendency to be dry and granular in appearance and in some cases yellowish pink longitudinal striations are visible. In most cases the subcutaneous and body fat has an obvious pink discoloration. WMD lesions are seen in the hearts of the more chronic cases.

Field trials. CONGENITAL WHITE MUSCLE DISEASE. When this disease was first recognized and in such serious proportions an immediate attempt was made to control the losses in those ewes yet to lamb (6). Three groups of 400 ewes 1–3 weeks off lambing were treated as follows: *1*) 5 mg Se as sodium selenate given once by mouth; *2*) 300 mg *dl*-α-tocopheryl acetate given once by mouth; *3*) controls.

All lambs born dead or dying within a few days of birth were examined for the presence of WMD. Table 1 shows that selenium given at such a late stage significantly reduced the losses from congenital WMD while α-tocopherol had no beneficial effect. Subsequent trials concerned with the prevention of congenital WMD are recorded later in this paper in the section dealing with ewe infertility.

(DELAYED) WHITE MUSCLE DISEASE. During the same year as the above trial we investigated the protective action of selenium and α-tocopherol on properties where the delayed form of WMD had occurred the previous year (6). On each property lambs were divided into three equal groups when from 2–3 weeks of age. One group received 1 mg selenium either by mouth or by subcutaneous injection, 1 group received 300 mg *dl*-α-tocopheryl acetate and one group served as control. Table 2 shows that α-tocopherol greatly reduced the incidence of WMD, while selenium almost completely prevented it.

WHITE MUSCLE DISEASE IN HOGGETS. A controlled trial carried out in 1954 (11) showed that drenching of hoggets with 500 mg α-tocopheryl acetate at 14-day intervals over a period of 3 months protected them from the development of myopathy following driving. During the last 2 years several controlled selenium trials have been carried out on properties affected with hogget WMD in previous years. Unfortunately insufficient numbers of sheep have become affected to evaluate the possible protective action of selenium.

Cattle

In New Zealand, dairy cows are kept out of doors the whole year round. They are mated in early summer to calve down in late winter. Their basic diet consists of lush rye grass and white clover pasture, supplemented in summer and winter with home grown silage, hay and brassica crops. Beef cattle are largely confined to the North Island and are run in conjunction with sheep in order to help to control the spring and autumn flush of pasture. Beef cows calve down about 2 months later than the dairy cows.

Field observations. WHITE MUSCLE DISEASE IN CALVES. This entity has been recognized at a low incidence in beef calves on not more than six pumice properties in the North Island, on all of which WMD also occurs in lambs. No cases have so far been reported in calves in the South Island, probably due to the fact that few cattle are raised on the improved pastures in conjunction with sheep in that Island. Calves are affected at from 1 to 4 months of age, they rapidly become lethargic and stiff, followed by recumbency and death in severe cases. Occasional sudden deaths have occurred during mustering. One outbreak occurred on a North Island sand country farm in a small group of dairy heifers which

TABLE 2. *Cases of White Muscle Disease*

Selenium Groups	Vitamin E Groups	Controls
2	21	50

[1] For references see J. R. Schubert et al., this symposium.

were being liberally dosed with cod liver oil. Post mortem findings differ in no way from those observed and reported in the U.S.A. and elsewhere.

Pigs

Most of the pigs in New Zealand are kept on dairy farms where they utilize the by-products of the butter and cheese industries. Their basic diet is whey and skim milk with various cereal supplements.

Field observations. HEPATOSIS DIAETETICA. During the last 3 yr this condition has been recognized in at least 20 piggeries scattered throughout both Islands (W. J. Hartley and R. M. Salisbury, unpublished data). Most, but not all, outbreaks have occurred in areas where selenium-responsive diseases are seen in sheep and most have occurred in the period of May to October. Outbreaks have been seen in piglets from 6 to 14 weeks of age mostly occurring either just prior to weaning or shortly after transference to fattening pens. Losses have varied considerably; in some instances up to 50% of susceptible litters have succumbed. In two big piggeries the losses continued over several months. On most affected properties the diet consisted of either whey or skim milk supplemented with barley meal and usually included cod liver oil, sometimes in liberal amounts. Several outbreaks have been complicated by salmonellosis.

Clinical signs are seldom seen, animals usually being found dead. When seen alive affected pigs are lethargic, they rapidly become prostrate and show labored breathing. In one outbreak affected animals showed stiffness, trembling and ataxia (5). Post mortem findings are quite characteristic. In some cases there is a generalized subcutaneous edema and in occasional cases the carcass is jaundiced. Skeletal musculature is sometimes paler than normal. In almost all cases there are copious amounts of clear straw colored fluid often with strands of fibrin in body cavities and sometimes edema of the mesentery. Liver lesions are a constant finding. In the peracute cases the liver is enlarged, congested and friable, with a mosaic appearance consisting of groups of bright red lobules. In other cases the mosaic appearance is more colorful, some groups of lobules being dark red, some bright red and others pale pink. In older standing cases, in addition to the focal multilobular lesions, there may be extensive focal fibrosis and fissures. In about 50% of cases the heart shows multiple minute white longitudinal streaks scattered throughout the myocardium. The lungs show congestion and edema.

Field trials. No controlled field trials have been carried out. However, except where salmonellosis has been a complicating factor, losses have ceased a few days after discontinuing the feeding of cod liver oil and giving the piglets 5 mg of selenium orally. Losses have also been controlled by selenium administration when no cod liver oil was being fed.

TABLE 3. *Selenium in Control of Exudative Diathesis*

	Ration		
	Control	ED producing	ED prod. + Se
% Incidence of exudative diathesis	0	25	1
% Mortality to 56 days	2	26	12
Average weight at 56 days, g	790	647	553

Poultry

The poultry industry is expanding rapidly in New Zealand and is run on similar but less intensive lines to that in other countries. New Zealand is not self supporting in wheat and most of the grain produced is grown in the South Island. We thus find that the South Island poultry feedstuffs contain South Island wheat, whereas those in the North Island contain imported Australian wheat.

Field observations. EXUDATIVE DIATHESIS AND MYOPATHY IN CHICKS. This was first recognized as a specific entity in chickens in 1951 (18). In that year all outbreaks occurred in the South Island of New Zealand and were associated with the inclusion of spoiled, South Island grown wheat in the diet. On many farms cod liver oil was also fed. From 1952 to 1959 only sporadic cases of exudative diathesis (ED) were reported; however, in 1960 many outbreaks were seen in the South Island and all were associated with South Island grown wheat. This year the grain was not spoiled and no cod liver oil was added to the diet.

Most outbreaks occur in chicks between 3 and 6 weeks of age. They become dejected, lose condition, show leg weakness, and the badly affected ones become prostrate and die. Invariably they show a red or greenish blue discoloration and swelling of the subcutaneous tissue, particularly under the wings and down the thighs. In a few outbreaks ED was also associated with nutritional encephalomalacia. Post mortem examination shows generalized subcutaneous blood stained edema, pale musculature and sometimes large pale areas in the gizzard musculature.

WHITE MUSCLE DISEASE IN PULLETS. During 1960 several outbreaks of WMD in 4–6 months old pullets were recognized in the South Island (R. M. Salisbury, and W. S. H. Poole, personal communication). Affected birds become listless, show a diminished appetite, loss in weight and decreased egg production. On post mortem examination the breast musculature is much paler than normal and contains many white longitudinal striations.

Field trials. EXUDATIVE DIATHESIS. Trials reported in 1953 showed that the addition of α-tocopherol to the diet greatly reduced the incidence of this disease (18). During 1960 a controlled trial was carried out on a Departmental poultry farm using an implicated South Island, ED-producing ration (R. M. Salisbury, and W. S. H. Poole, personal communication). Three groups of 100 4-day-old chicks were given the following treat-

ments: *1*) control ration of high quality; *2*) South Island ED ration; *3*) South Island ED ration with selenium. The selenium was given as sodium selenate equivalent to 15 mg of selenium per gallon of drinking water, intended to be given for 1 day only. By mistake the chicks actually received it for 3 days. It is seen (Table 3) that the addition of selenium almost completely prevented ED (the reduced weight gain in the selenium group can be explained by a combination of apparent mild selenium toxicity and an outbreak of black head). Several controlled treatment trials have been carried out subsequently on properties experiencing outbreaks of ED. In every case 15 mg of selenium added to 1 gallon of drinking water for 1 day immediately controlled the disease and many clinically affected birds made a rapid recovery.

Horses

The two conditions about to be described are not definitely known to be responsive to selenium administration, as, for obvious reasons, it is impossible to carry out controlled trials with thoroughbreds. However, they have features in common with the myopathies already described and veterinarians claim that selenium is useful both for prevention and treatment. In New Zealand, thoroughbreds, except while in training, are kept in the main on pasture. The brood mares foal in the spring and early summer and they run with their offspring on lush rye grass and clover pastures. Little or no supplementary feed is given to the mare or foal.

Field observations. WHITE MUSCLE DISEASE IN FOALS. This condition has been recognized in several large thoroughbred studs in the North Island (4, 11). Unaccountably it has occurred only on the heavier type of soils and in areas where selenium responsive diseases in sheep and calves have not been reported. It occurs during spring and summer in suckling foals from a few days to 5 months old, running with their dams at pasture, and is apparently unassociated with excessive exercise.

Foals may be affected with WMD from 3 days to 5 months of age. Clinically they show rapidly developing dejection, increased respiration, stiffness of gait, inability or unwillingness to suckle, prostration and death within 1–7 days. Some mildly affected ones recover spontaneously. Many affected animals show a pronounced painful swelling of the subcutaneous tissues particularly beneath the mane but also over the gluteals and in the inguinal region. Many cases show salivation associated with extensive desquamation of the epithelium of the tongue and some show a board-like firmness of some muscles. The urine may be coffee-colored. On post mortem examination there is a bilaterally symmetrical palor of groups of skeletal muscles together with swelling and yellow-reddish-brown discoloration of subcutaneous and body fat.

"TIED-UP" RACEHORSES. This condition is quite commonly seen in thoroughbreds in New Zealand. It occurs during training in horses on high carbohydrate diets and is associated with exercise following a spell of a few days on full rations. It is characterized by shivering, disinclination to move, muscle stiffness and tenderness and in some cases the passage of a dark red-brown urine.

TABLE 4. *Empty Ewe Incidence and Congenital White Muscle Disease*

Congenital WMD	Total No. Properties	Empty Ewe Incidence, (%)		
		10	10–30	30
Present	21	3	8	10
Absent	16	12	4	0

TABLE 5. *Lambing Data—3 Trial Farms, Rotorua-Taupo Area*

Owner	Lambing %		Empty Ewe %		WMD %	
	Control	Se	Control	Se	Control	Se
Evans	70.5	93.6	26.1	6.4	12.2	0
Carrington	60.6	94.9	31.1	7.5	37.5	0
Dick	53.8	92.5			22.2	0

INFERTILITY IN SHEEP

Field observations. In some areas experiencing white muscle disease in lambs, particularly the congenital form, there is in some seasons an associated barren ewe problem. Table 4 analyzes the incidence of barren ewes in 1958 on 37 South Island properties which had experienced congenital and/or delayed WMD in lambs the previous year. It is seen that of the 21 properties once again experiencing WMD in 1958 almost half had a barren ewe incidence of over 30%. On most affected properties the ewes are in good condition at mating and the exact cause for the barrenness is not known; ewes are served and apparently conceive but do not produce a lamb at term. Ewes barren one year and treated with selenium the next have produced close to 100% of lambs.

Field trials. In 1959 controlled selenium trials were carried out on 11 properties which for several years previously had experienced abnormally low lambing percentages (12). White muscle disease had occurred in lambs on all properties, and on at least three there existed a severe form of selenium responsive unthriftiness and mortality in lambs. On these farms half of the trial ewes (from 100 to 500) were given 5 mg selenium as sodium selenite by mouth at monthly intervals from 1 month prior to mating until just before lambing. The control ewes were separated from the dosed ones at lambing. All dead lambs in both groups were subjected to a detailed post mortem examination with particular reference to the presence of WMD. Table 5 gives the results of the three trials carried out on North Island pumice soils. It shows that selenium greatly increased the lambing percentage, this being largely due to a reduction in the number of barren ewes. The lamb mortality up to tailing (3 weeks)

in the three groups varied from 11 to 14% in those dosed with selenium and from 15 to 23% in the controls. Table 5 also shows that selenium completely prevented the development of congenital WMD (no delayed type occurred in these trials). In approximately half of the cases the WMD lesions were probably not sufficiently severe to account for the death of the lamb. Similar, though less spectacular results were obtained for the eight trials carried out on the South Island farms.

Some interesting data were obtained from uncontrolled trials carried out in the same season on 14 recently settled contiguous pumice farms (Dick's farm in Table 5 was on this block of country). On six of these properties 5 mg Se was given once only to half the ewes 2–4 weeks before mating, on one property all the ewes received 2½ mg Se once only prior to mating, and on seven properties no selenium was given. Table 6 gives the lambing percentages for the various groups and includes those for the farm on which the controlled trial was carried out. This table also includes the lambing percentages for the previous two seasons and also for the 1960 season—the first year of general selenium administration. The results for this uncontrolled trial showed that one dose of selenium given prior to mating apparently substantially increased lambing percentages: however, it may not have prevented congenital WMD.

As monthly administration of selenium is impracticable under New Zealand farming conditions and in view of the results obtained from the uncontrolled trials reported in Table 6, more detailed investigations were carried out in 1960 to ascertain a more practicable and yet still effective method of selenium dosing. This trial was carried out on a large, state owned block of country bordering on the block referred to in Table 6. This land was being developed from virgin scrub and for the past 2 yr had had lambing percentages of 36 and 46%; it also had a selenium responsive unthriftiness in lambs. The ewes used in this trial had had two crops of lambs on the property. They were divided into six even groups of 200 and subjected to the following treatments: *1*) 5 mg Se by mouth monthly from 1 month prior to the commencement of mating till just prior to lambing. *2*) 5 mg Se 1 month prior to mating, repeated 2 months later and 1 month prior to lambing. *3*) 5 mg Se once only 1 month prior to mating. *4*) 25 mg Se 3 times as in group 2. *5*) 25 mg Se once as in group 3. *6*) Controls.

TABLE 6. *Lambing Percentages on Oruanui Block, Wairakei Average Lambing %—1959–60 (15 Properties)*

No. of Properties	Pre-Tupping Ewe Treatment		
	No Se	Half Se	All Se
7	52.2 (35.0–74.0)		
6		72.4 (61.5–77.0)	
1			85.7
Trial farm	53.8		92.5

Avg lambing % 11 properties, 1957–58 66.2 (31–86)
Avg lambing % 15 properties, 1958–59 81.2 (60–97)
Avg lambing % 7 properties, 1960–61 100.0 (86–108)

TABLE 7. *Effect of Selenium on Ewe Fertility at Taupo (200 Ewes/Group)*

Treatment	Lambing % At Docking	Congenital WMD % Incidence	Barren Ewe % Incidence
5 mg Se monthly	116	0	4
5 mg Se 3 times	112	0	2.5
5 mg Se once	118	12	5
25 mg Se 3 times	108	0	3
25 mg Se once	117	0	3
Controls	74	16	30

The 1,200 ewes were mated and run together until just before lambing when they were divided into their respective groups and lambed separately. It is seen (Table 7) that all selenium treatments substantially increased the lambing percentages and that there was no significant difference between treatments. Although the incidence of congenital WMD was very low, it was evident that 5 mg Se, given once only, was insufficient to prevent it.

In all the ewe fertility trials described in this paper the ewes, at the commencement, were in average to good condition. However, at lambing the dosed ewes appeared to be more thrifty, produced more milk and had heavier and livelier lambs than did the controls. Further, ewes dosed with selenium produced up to 2 lb (20%) more wool.

SELENIUM RESPONSIVE UNTHRIFTINESS IN LIVESTOCK

Sheep

Failure to thrive is probably the most important cause of wastage in the New Zealand sheep industry. Although some of the unthriftiness is directly associated with under feeding, most of it occurs in young stock grazing on abundant, apparently good quality pasture. Known causes of unthriftiness are cobalt deficiency and internal parasites. However, it is commonly accepted that these entities account for only a small part of the unthriftiness experienced. A further type of unthriftiness termed "autumn-flush hogget illthrift" is seen in the North Island on pastures stimulated by warm early autumn rains (1). Accumulating information suggests that much of the previously unclassified unthriftiness in lambs, other than the "autumn-flush hogget illthrift," responds to selenium administration.

Field observations. SELENIUM RESPONSIVE UNTHRIFTINESS IN YOUNG SHEEP. This condition typically occurs on improved farms situated on light, readily leached types of soil, e.g., stones, silt, sand and pumice, and rarely on the heavier soils. Fig. 1 outlines the main areas involved; from this it will be seen that, in addition to occurring in lamb WMD areas, selenium response unthriftiness (SRU) also occurs in areas where WMD has not been recognized. Although SRU is largely confined to lambs and hoggets, in some areas and in

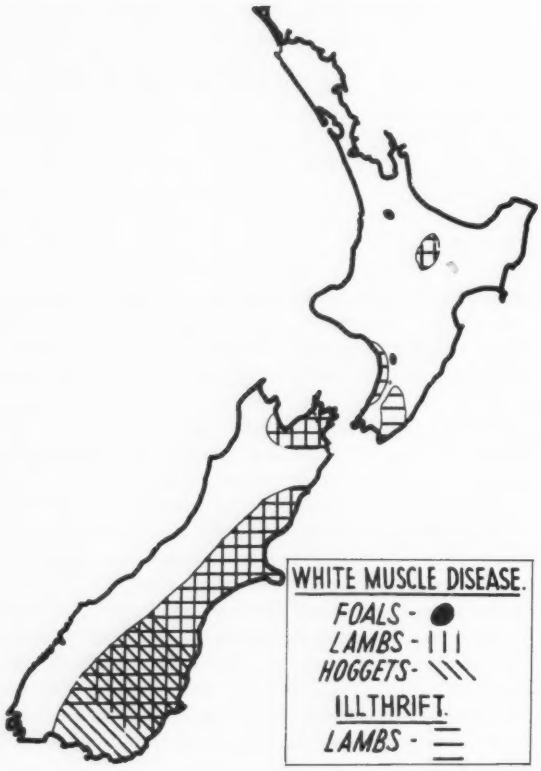

FIG. 1. Distribution of selenium responsive myopathies in New Zealand.

TABLE 8. *Effect of Selenium on Lamb Unthriftiness (Rotorua-Taupo)*

Location	Lambs Per Group	No. Deaths		Avg. Wt. Gain, lb	
		Control	Selenium	Control	Selenium
1. Rerewhakaaitu	25	13	8	5.9	11.1
2. Reporoa	25	5	1	21.8	29.6
3. Reporoa	25	15	2	4.8	15.6
4. Broadlands	25	3	1	14.1	25.1
5. Wairakei	21	2	2	19.2	22.3
6. Wairakei	20	8	1	10.3	14.5
7. Wairakei	36	5	1	12.8	20.7
8. Wairakei	24	4	0	3.7	17.8
Totals	201	55	16		

some seasons adult stock are also involved. On affected country lambs may thrive until 2 or 3 months of age, they then stop growing, lose weight, become dejected, stop eating and die. The wool of affected lambs loses its character and some sheep develop a diarrhea. Deaths from SRU continue throughout the summer and autumn but usually cease by midwinter.

Lambs with SRU on post mortem examination show advanced emaciation and soft brittle bones. In some cases the remaining fat is yellow in color and many carry a very high gastrointestinal worm burden. Affected lambs produce less wool (up to 30%) of poorer quality than do the selenium dosed ones. When WMD and SRU occur on the same property in one season, unthriftiness usually does not become apparent for several weeks and sometimes months after the myopathy.

Field trials. SELENIUM RESPONSIVE UNTHRIFTINESS IN LAMBS. Trials carried out in unweaned thrifty lambs showed that selenium had a significant growth promoting effect (16). A similar, but more pronounced effect was obtained when selenium was given to lambs in flocks where there was a severe form of unthriftiness and mortality (7, 13). Table 8 gives the results of trials carried out on eight badly affected North Island pumice properties. Altogether in the 1958–59 season 33 controlled trials were carried out on unthrifty weaned lambs in the central and southern part of the North Island and in 22 of these a statistically significant weight gain was obtained in the selenium-dosed groups. In these trials 5 mg of Se as selenate or selenite was given monthly by mouth.

During the 1959–60 summer the New Zealand Department of Agriculture organized a dominion-wide survey to ascertain the distribution of SRU in lambs. Two hundred and seventy-four trials, approximately equally divided between both Islands, were carried out mostly on unthrifty weaned lambs on all classes of soils. The results of this survey show that there was a statistically significant response to selenium on 43% of the South Island properties and 14% of the North Island ones (P. B. Lynch, and T. G. Robertson, personal communication). Most of the responses to selenium occurred on the light soil types; however, a few occurred on the heavier soils, but none on peat. This survey is being repeated this summer.

Recent and current selenium trials have been designed primarily to ascertain an effective, safe, and at the same time convenient, method of selenium administration. Up to the present all our trial work has been concerned with individual dosing of selenium either by the oral or subcutaneous routes. Application of selenium to pasture will be investigated next season. One detailed series of trials has been carried out on the North Island pumice

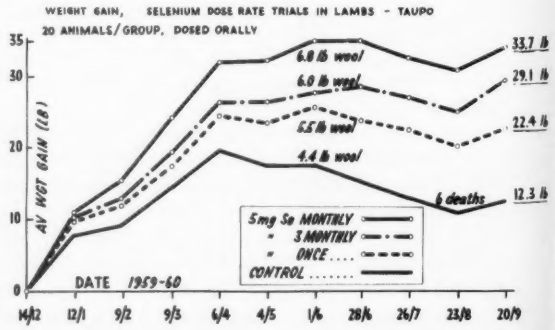

FIG. 2. Effect of selenium dosage on unthrifty lambs (pumice soil, North Island).

soils—where there is a barren ewe problem, and WMD, and a serious SRU in lambs—and the other on the North Island coastal sand where there is no barren ewe problem, and no WMD, but a serious SRU problem in lambs.

The trials on the pumice have shown that:

1) Lambs born from selenium-dosed ewes grew faster than those born from undosed ewes.
2) Lambs born from selenium-dosed ewes and themselves dosed at tailing (2–3 weeks) grew faster than those similarly treated but born from undosed ewes.
3) Lambs responded equally to monthly doses of 1.25, 2.5 and 5.0 mg Se given either by the oral or subcutaneous routes.
4) Monthly dosing gave slightly better growth rates than 3 monthly dosings (Fig. 2).
5) Ten and twenty milligrams of selenium given monthly by mouth had an apparent slight toxic effect as evidenced by decreasing growth response.

The trials on the sand have shown that:

1) Lambs responded only slightly to oral selenium from tailing (30/8/60 to 27/10/60). However, during November and December—the period of maximum pasture growth—there was a marked response.
2) Five milligrams of selenium given orally at tailing (30/8/60) and repeated at weaning (25/11/60) and shearing (17/2/61) resulted in a weigth gain, up to 17/3/61, identical to that obtained from 5 mg of selenium given orally at monthly intervals from tailing, and slightly better than that obtained from 1 mg selenium given orally at tailing, weaning and shearing (Table 9).
3) There was no difference in growth response to dose levels of selenium between 0.63 and 5.0 mg when given by mouth at 14-day intervals, and no difference between 5 mg monthly and 5 mg fortnightly. One oral dose of 10 mg produced a satisfactory growth response for 18 weeks (Fig. 3).
4) Weaned lambs responded equally for 14 weeks to one oral or subcutaneous dose of selenium at levels from 0.63 to 5.0 mg.
5) 300 mg *dl*-α-tocopheryl acetate given either by mouth or by subcutaneous injection at monthly intervals for 3 months did not give a weight gain above that of the undosed selenium responsive controls.

TABLE 9. *Effect of Selenium on Lamb Unthriftiness—1960–61*

Selenium Treatments			Average Total Wt. Gain, lb 30/8–17/3	% Mortality From Unthrift 25/11–17/3
Docking 30/8	Weaning 25/11	Shearing 17/2		
1. 5 mg monthly from 30/8			42.2	0
2. 5 mg	5 mg	5 mg	44.4	0
3. 1 mg	1 mg	1 mg	40.6	0
4.	5 mg	5 mg	40.1	0
5.	5 mg		38.7	0
6.	1 mg	1 mg	35.5	5
7.			33.0	40

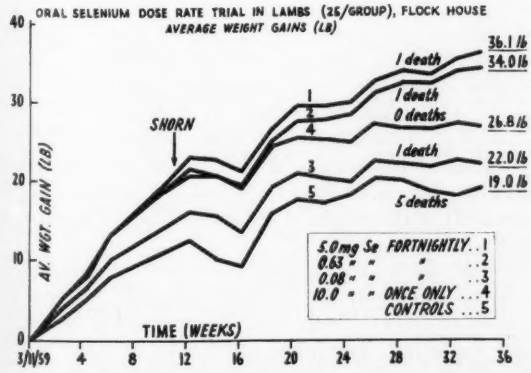

FIG. 3. Effect of selenium dosage on unthrifty lambs (sandy soil, North Island).

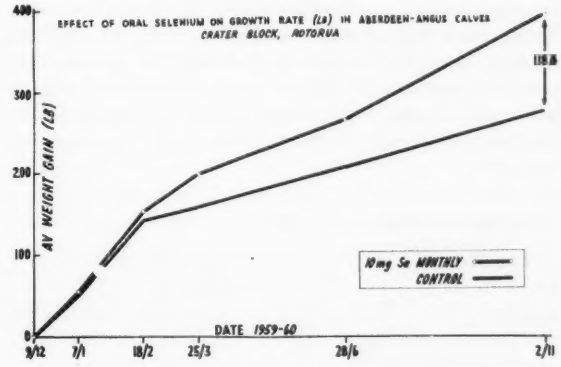

FIG. 4. Growth effect of selenium on unthrifty calves (pumice soil, North Island). *Upper curve:* 10 mg selenium monthly by mouth.

Cattle

Unthriftiness in cattle is widespread in the North Island but is not such an important cause of wastage as it is in sheep. Cobalt and copper deficiency and parasitism apparently play only a limited part in the causation of the problem.

Field observations. SELENIUM RESPONSIVE UNTHRIFTINESS IN CATTLE. In some seasons, particularly those associated with lush growth, dairy calves situated on the coastal sandy soils and stony soils of the south of the North Island and beef and dairy calves on the central pumice soils may show a severe and rapidly progressive form of unthriftiness which is usually associated with a profuse diarrhea. This condition largely occurs in the autumn and winter months and on some farms may be associated with a heavy mortality. Occasionally similar outbreaks occur in adult cattle.

Field trials. SELENIUM RESPONSIVE UNTHRIFTINESS IN CATTLE. Initial field trials (Fig. 4) have shown that diary and beef calves on pumice soil respond to selenium

administration (14; Hartley, unpublished observations). These trials have been extended to find the smallest dose given at the longest interval which is necessary to produce satisfactory weight gain. Preliminary results from our current trial suggest that a subcutaneous injection of 20 mg Se given every 4 months may be sufficient.

PARADONTAL DISEASE OF SHEEP

This disease is an important cause of unthriftiness and losses in some areas of New Zealand (9, 17). It is included in this paper in view of its apparent association with proven selenium responsive diseases. The pathology of paradontal disease has been described in detail (15). Briefly, there is loosening and shedding of permanent premolars and molars and sometimes also of incisors, associated with extensive paradontal fibrosis and sometimes with gingival and alveolar infection.

One of us (*WJH*) recently carried out a limited survey of farms where paradontal disease had been a serious problem. On the majority of properties it was found that SRU or WMD in lambs or ewe infertility had also occurred and that, on the properties where selenium had been given to the ewes, the disease had virtually disappeared. Long term trials are in progress to ascertain if selenium will protect ewes from the disease.

LABORATORY INVESTIGATIONS

Selenium determinations. Two fluorometric methods for the determination of micro amounts of selenium in plant and animal tissues have been developed in New Zealand (2, 19). Both have recently been modified (F. B. Cousins, personal communication; A. B. Grant, personal communication).

Selenium content of organs from sheep affected with selenium responsive diseases. Livers and kidneys have been examined from lambs affected with the delayed type of WMD and have been shown (Table 10) to contain significantly less selenium than those from unaffected lambs (3). In a few samples analyzed to date, the same workers have not been able to demonstrate a significant difference in selenium levels between undosed selenium responsive unthrifty lambs and undosed nonselenium responsive unthrifty lambs, nor between hoggets affected with WMD and normal hoggets. Large numbers of organs from all conditions referred to in this article are in cold storage awaiting analysis.

TABLE 10. *Selenium Content (ppm) of Tissues From White Muscle Disease Lambs*

	Normal		WMD	
	Kidney (16)	Liver (13)	Kidney (16)	Liver (15)
Mean Se conc.	2.22	0.28	1.13	0.18
Range	1.2–3.6	0.13–0.54	0.90–1.50	0.07–0.29

TABLE 11. *Selenium Uptake of Mixed Pasture Fertilized With Selenium*

Selenium Applied As:	Period—Application to Sampling	Rate of Application, oz/acre					
		1	2	4	8	16	32
		Se content of pasture (ppm)					
Selenate	6 weeks	5.9	11.6	28.0	70.1	169.0	360.0
	10 months	0.17	0.27	1.0	1.09	2.6	7.2
Selenite	6 weeks	1.1	2.8	5.0	9.0	11.5	20.0
	10 months	0.23	0.31	0.21	0.21	0.45	0.92

Selenium content of pasture. Many pasture samples have been collected both from properties affected and unaffected with SRU in lambs. Preliminary results indicate that SRU pastures from North Island trials are in general much lower in selenium (<0.03 ppm Se) than those from unresponsive pastures (0.03–0.14 ppm Se). No apparent correlation, however, exists for South Island pastures. Samples taken from a small number of South Island WMD pastures show selenium levels between 0.02 and 0.05 ppm Se.

Selenium uptake of mixed pasture following application of increasing rates of selenium. Selenium at concentrations from 1 oz to 2 lb per acre, as sodium selenate and selenite, was applied to plots on a closely grazed mixed pasture—presumed to be selenium unresponsive—at Wallaceville. Table 11 indicates the selenium levels after 6 weeks and 10 months.

Trials carried out on a selenium responsive property on the North Island pumice showed that there is a differential selenium uptake between common pasture species following application of varying concentrations of inorganic salts. White clover took up amounts of selenium considerably less than the grasses. (E. B. Davies, and J. H. Watkinson, personal communication.)

HISTOPATHOLOGY

A comprehensive series of specimens has been taken from all the selenium-responsive and allied myopathies discussed in this paper. The tissues have been fixed in 10% formalin, routinely stained with hematoxylin and eosin and as the occasion demanded, by Kossa's method for calcium, van Gieson's method for fibrous tissue, Mallory's phosphotungstic acid hematoxylin method for muscle and Wilder's method for reticulum. As the histological picture in all the above described diseases is similar, a description of the lesions will be given under the organs or tissues affected. Table 12 gives an assessment of the extent of the myopathy in the various entities.

Skeletal muscle. In the peracute cases of myopathy, there is commonly a universal massive coagulative necrosis of muscle fibers with fragmentation which is often segmental. This stage is followed by lysis of the necrotic fiber or by calcification, the latter often apparently starting in the myofibrils. At a later stage there is

TABLE 12. *Specific Myopathies of N. Z. Livestock Responsive to Selenium*

	Distribution of Lesions				
	Myo-necrosis		Hepatic Necrosis	Steatitis	Exudative Diathesis
	Cardiac	Skeletal			
Sheep					
Congenital WMD	++	±	−	−	−
Delayed WMD	++	++	−	−	−
Hogget WMD	±	++	−	−	−
Cattle					
Calf WMD	±	++	−	−	−
Pigs[1]					
Hepatosis Dietetica	+	±	++	−	−
Horses					
Foal WMD	−	++	−	+	−
Poultry					
Chick ED	−	±	−	−	++
Pullet WMD	−	+	−	−	−

extensive sarcolemmal nuclei proliferation and later still regeneration of muscle fibers. In some cases one muscle may show relatively oldstanding lesions while another may show very recent lesions. Further, it is not uncommon in some entities to see recent and old lesions in the same section of muscle. The muscle lesions in ED differ in no way from those described above except that calcification is usually not extensive and that, in the acute cases, there is often extensive interstitial edema, congestion and sometimes hemorrhage. The gizzard musculature shows similar lesions to those seen in the skeletal musculature.

Skeletal muscle lesions indistinguishable from those described above have been seen in isolated cases of several other totally unrelated specific disease entities in which the myopathy has been an incidental and unexpected finding.

Cardiac muscle. In recent severe cases the microscopic lesions consist of extensive areas of, usually, subendocardial (lambs and calves) or focal disseminated (pigs) coagulative myonecrosis. Purkinje fibers are usually, but not invariably spared. Most cardiac lesions appear to be of the same duration. In older cases the affected musculature may either become calcified or lyse and be replaced by macrophages and fibroblasts. In very oldstanding cases the lesion may consist of large calcified plaques.

Liver. Significant hepatic lesions have been encountered only in the pig and these consist of focal multilobular areas of hepatocellular necrosis. In recent severe cases this is seen as a complete or almost complete lobular coagulative hepatocellular necrosis and sinusoidal congestion. In some cases a narrow rim of perilobular parenchyma is spared. This is followed by lysis of most of the necrotic material and replacement by hemorrhage and usually calcification of any remaining parenchyma. If the animal survives the initial injury the affected lobule collapses and is invaded by macrophages and fibroblasts from Glisson's capsule, and in oldstanding cases it may be replaced completely by fibrous tissue. In some outbreaks of hepatosis diaetetica it is not uncommon to see recent and oldstanding lesions in the same liver.

Adipose tissue. In WMD in foals the adipose tissues are the site of extensive lesions in all but the peracute cases. The earliest lesion consists of interstitial edema and congestion with focal neutrophile infiltration of both interstitial tissue and fat cells. At this stage many of the interstitial capillaries appear to be thrombosed. Older lesions show increased neutrophile invasion followed by extensive necrosis of the affected tissue leading in some cases to caseation and/or calcification. Ceroid pigment has not been seen in these cases.

Selenium-responsive infertility in sheep. So far no attempt has been made to study the pathology of the reproductive tract in this condition.

Selenium-responsive unthriftiness. A large series of specimens taken from SRU in lambs are at present being examined. Preliminary results indicate that there are no striking pathological changes.

CONCLUSION

We have, in New Zealand, recognized all the recorded selenium-responsive diseases of livestock; in addition we have observed several that have not been described elsewhere. The investigations reported in this paper indicate that selenium-responsive diseases are widespread throughout the dominion and that they are of considerable economic importance, particularly to the sheep industry. We still do not know whether the diseases described are associated with a deficiency of selenium, per se, in the food being consumed. In this respect, it must be remembered that different organic compounds of selenium are very different in their potency, and that inactive forms of selenium have been shown to occur in nutrients. Analytical determination of selenium in feeds does not measure their protective potency against Factor 3—selenium deficiency. We do know, however, that in at least one entity—delayed WMD in lambs—the tissues contain significantly less selenium than normal.

The presence of widespread severe selenium responsive unthriftiness in some areas in the absence of white muscle disease and ewe infertility, and the apparent infrequent occurrence of SRU in some areas experiencing WMD and ewe infertility, indicates that other factors may interfere. It seems possible that pasture in some areas of New Zealand may contain a dystrophogenic factor and also a factor which interferes with fertility. Further, the liberal use of gypsum might contribute to the development of selenium deficiency, because sulfate is known to inhibit selenium utilization in plants and bacteria.

Our immediate investigations are concerned with

ascertaining the minimal physiological amount of selenium required to prevent and control the diseases described. By doing this, we expect to afford optimal protection from these diseases and to warrant the Factor 3- selenium content of animal tissues to be adequate and normal.

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Experimental results with selenium in white muscle disease of lambs and calves[1]

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WHITE MUSCLE DISEASE (WMD) of lambs and beef calves has accounted for substantial and increasing economic losses in Oregon since its recognition over 30 years ago. This myopathy is prenatally influenced by the dams' consumption of leguminous forages grown in reasonably well-defined areas. The disease involves degenerative changes in cardiac and skeletal muscles to varying degrees, with extensive calcification a frequent and striking characteristic (Figs. 1–5). The nature and incidence of WMD being described (13) lent impetus to the search for adequate control measures, with emphasis on prophylaxis. Lack of a clear definition of the role of vitamin E in the disease quickly led to further investigations (5, 15, 20, 24) on the effectiveness of Factor 3-active selenium, since it had been shown that the two dietary factors were simultaneously lacking in other syndromes (19, 22, 23). This report deals with early observations and subsequent investigations of WMD as encountered under Oregon conditions.

Precise details of year by year experimental conditions have, for the most part, been reported (14, 15, 16, 17, 18). In general, mature Hereford cows and crossbred ewes were maintained on pasture, summer through early fall, having been bred during this period. Both species were approximately one-third advanced through gestation (ca. Nov. 1) when allotted in confined groups of 10–12 and bled for analysis. The dams were routinely bled again about February 1, which constituted a late-gestation sampling. A terminal post-partum bleeding of ewes was performed at the conclusion of lambing (ca. April 1).

Lambs generally were bled at sacrifice when 6 weeks of age, although in some cases earlier, when clinically indicated. In certain instances of twinning, one of each pair was sacrificed when less than 1 week old. Calves were routinely sacrificed at 8 weeks of age, although some died earlier. Additional data were obtained from a limited number of blood samples taken within 24 hours of birth. Evidence of gross lesions of the skeletal musculature and heart was sought at necropsy. Blocks of tissue were routinely taken for histopathological examination (14) and for chemical studies.

[1] Technical Paper No. 1399, Oregon Agricultural Experiment Station.

Blood determinations included plasma total tocopherols (25), calcium and magnesium, as well as whole blood phosphorus and hemoglobin (5). Muscle tissues were analyzed for calcium, magnesium, sodium and potassium (3, 6). Forages were variously analyzed for their contents of calcium and phosphorus (12), total (1) and inorganic (2) sulfur, tocopherols, molybdenum and selenium.

The animals were group fed in dry-lot pens, the cows receiving 20 lb forage per head daily and the ewes 4 lb. Control rations, obtained from areas where WMD has not been encountered, consisted of grass hay and silage, with the exception of 1957–58 when hays were used solely, alfalfa for cows and grass-Ladino clover for ewes.

The basal ration for both cows and ewes during 1953–54 and 1954–55 consisted of a cull bean-alfalfa regimen (26), the hay being purchased on the open market from a general area where considerable WMD had occurred (5). The basal forage for cows thereafter was second-cutting alfalfa hay, successive years' crops being obtained from a specific ranch having a long history of WMD incidence (18). Basal forage for ewes from 1955–56 to date has been Ladino clover chaff and/or clippings from an area where WMD is similarly prevalent (17). All ewes were supplemented with ground oat grain at the rate of ¼ lb/head/day, from a lot having been demonstrated in the first year to be innocuous with regard to WMD incidence. Various treatments are indicated in the results (Tables 1, 2 and 4) and in the chronological discussion following.

The original objective, as a basis for these studies, was to reproduce WMD by feeding native forages under controlled conditions. Two years experience (1953–54 and 1954–55) with both cows and ewes fed an alfalfa hay-cull bean ration resulted in only a single incident. Data from these trials, however, provide a partial frame of reference by which to judge subsequent results, in particular among calves.

A severe outbreak resulted the following year when the basal ration of alfalfa hay fed to cows was obtained from a ranch where WMD had been a common occurrence. Of nine apparently thrifty calves born, four died spontaneously and showed extensive cardiac lesions. At

sacrifice, three additional cases were evident from gross, predominantly skeletal muscle lesions. When, however, forage from two successive years' crops from the same field comprised the sole basal ration, WMD incidence diminished to two cases in the first year, detected histologically, and to none in the second.

FIG. 1. Six-week old calf, affected with WMD 20 days.

FIG. 2. Experimental WMD in lamb: hind limbs affected.

FIG. 3. Cross section of 46-day-old lamb leg showing affected muscle (right) and gross fat replacement.

FIG. 4. Lamb heart, field case, showing extensive subendocardial myopathy.

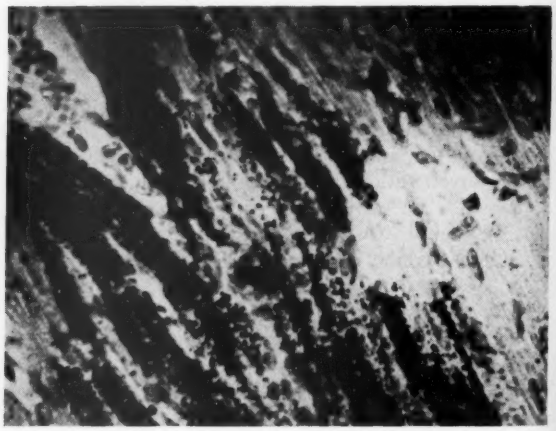

FIG. 5. Lamb heart, showing calcification at birth (Van Kossa, × 245).

Reference to Table 1 reveals no consistent correlation between plasma total tocopherol levels and the varying incidence of WMD over 5 years, with particular reference to comparisons between control and basal groups within any given year. The same is apparent among values for plasma calcium and magnesium, with the latter a possible exception among the severely affected animals. Very low tocopherol levels were generally encountered among new-born animals, regardless of affectation with WMD.

Analyses of forages used in the cattle trials (Table 3) show no direct relationship between tocopherol content and WMD incidence, either between years or between control and basal. The wide calcium-phosphorus ratio previously noted for the high incidence year (18) warrants continued interest by virtue of its decline in succeeding year's crops. This may well be considered a contributory factor only, in view of doubt raised by

TABLE 1. *Bovine Plasma Tocopherol, Calcium and Magnesium Contents*[a] *as Related to WMD*

Plasma Value	Cows			Calves		WMD Incidence
	On trial	Late gest.	1 Day post-p.	1 Day	2–8 Weeks	
1953–54, Basal ration						
E[b]	297±31	188±8			94±8	0/11
Ca[c]		11.0±.2			12.1±.3	
Mg[c]		2.3±.1			2.0±.5	
1954–55, Control ration						
E	245±15	461±28			144±26	0/9
Ca	9.8±.3	10.2±.1			11.6±.3	
Mg	2.4±.1	2.2±.1			1.7±.1	
1954–55, Basal ration						
E	[d]	149±10	139±11		60±5	1/10[e]
Ca		9.6±.2	9.8±.1		11.3±.1	
Mg		2.3±.1	2.2±.1		1.7±.1	
1955–56, Control ration						
E	335±73	220±23	446±40	18±8		0/8
Ca		8.5±.3	9.8±.3	11.2±.3		
Mg		1.6±.1	1.9±.1	2.1±.1		
1955–56, Basal ration						
E	[d]	298±40	310±35	17±4	86±17	7/9[e]
Ca		9.2±.3	9.9±.3	11.2±.2	11.4±.2	
Mg		1.9±.1	2.2±.1	2.2±.2	1.6±.1	
1956–57, Control ration						
E	448±35	303±15	288±26	32±5	63±11	0/10
Ca	9.3±.1	10.2±.1	9.8±.2	11.2±.3	11.9±.1	
Mg	2.0±.2	2.3±.1	1.8±.1	2.3±.2	1.8±.1	
1956–57, Basal ration						
E	[d]	322±14	123±11	31±4	79±12	2/14[f]
Ca		10.3±.1	9.9±.1	10.6±.1	11.5±.2	
Mg		1.9±.1	2.1±.1	2.2±.1	1.9±.1	
1957–58, Control ration						
E	375±20	212±30			33±9	0/5
Ca	9.4±.3	10.2±.2			11.7±.3	
Mg	2.0±.1	2.0±.1			1.8±.1	
1957–58, Basal ration						
E	[d]	229±16			45±7	0/6
Ca		9.7±.3			11.7±.3	
Mg		2.1±.1			1.9±.1	

[a] Mean ± standard error. [b] Total tocopherols, μg/100 ml plasma. [c] mg/100 ml plasma. [d] Control values represent both groups when going on trial. [e] Diagnosis/necropsy: gross cardiac and skeletal WMD. [f] Diagnosis/histology: microscopic skeletal WMD only.

direct comparison with the 1957–58 control alfalfa forage.

Subsequent analyses have shown a surprising, direct rather than inverse, relationship between selenium content of the basal alfalfa and WMD incidence. The common practice of gypsum fertilization of alfalfa, because of increased yield benefits, resulted in analysis of the forages for their sulfur contents. The results shown confirm an earlier observation (2) that the level of sulfur fertilization of alfalfa is reflected by increasing contents of total sulfur, largely as a result of accumulation of sulfate. In turn, it has been shown (7, 9, 11) that where relatively large amounts of selenium have been at issue, sulfate may be antagonistic. Speculation that sulfate might interfere with plant uptake of essential amounts of selenium, or render it Factor 3-inactive therein, or interfere with its utilization by the animal, resulted in trials with sheep, to be described below. Although possibly an artifact of the level of sulfur fertility (4), the inverse relationship of molybdenum contents of the basal forages with WMD incidence in the calves is of interest.

During the year 1956–57, it was found that a high incidence of WMD in lambs could be produced experimentally by dams winter fed on Ladino clover chaff, a by-product of seed production which is commonly fed to stock (17). The consistency with which this could be done permitted a variety of experimental control measures to be superimposed on a WMD-inducing forage basal.

The original plan for the following year (1957–58) envisioned groups including animals fed control and basal rations, with vitamin E supplementation of additional ewes: orally at the rate of 100 IU per ewe daily;[2] and parenterally at the rate of 770 IU per ewe weekly.[3] The announcement (22) during the summer of 1957 of the identification of selenium as an integral component of Factor 3 led to the inclusion of this element in the basal ration for a fifth group of ewes, at the rate of 0.1 ppm Se, as sodium selenite, calculated for the entire ration. As in the case of vitamin E therapy, the supplementation continued through the last two-thirds of gestation and about 1 month post-partum. The results (14, 15) shown in Table 2 indicate the excellent degree of protection against gross WMD prenatally afforded by selenium but not by vitamin E. Although plasma total tocopherol levels were elevated in the dams treated with the vitamin, there was no benefit to the offspring. Nor was there any indicated relationship of incidence of the disease in the young to the tocopherol values obtained from all bleedings of all groups. Values for blood calcium, phosphorus, or, in the case of lambs, magnesium, are not indicated as criteria of WMD, while altered values for hemoglobin are probably only an indication of relative general well being.

In addition to seeking confirmation of the results of feeding selenium in the previous year, primary emphasis during 1958–59 was on treatment of the lambs. Consequently, of the groups of ewes fed, three subsisted on the basal forage alone. Among these, the lambs born received: no treatment; a drench of 2,000 IU vitamin E,[4] in equal doses at 1 and at 4 days of age; or sodium selenite in physiological saline injected subcutaneously, at birth and again at 2 weeks. The doses of 0.28 and 1.12 mg Se were calculated to provide the equivalent of 0.2 ppm of the estimated dry matter intake until necropsy when 6 weeks old. A fourth group of ewes again received a supplement of sodium selenite at 0.1 ppm Se in the ration throughout the trial period. The fifth group was a duplicate of the preceding, with the further addition of 0.1% sulfur in the ration as sodium sulfate. By analysis this elevated both the total

[2] *d*-α-Tocopheryl acetate, Myvamix, courtesy of D. C. Herting, Distillation Products Industries.

[3] *d*-α-Tocopheryl-PGS, courtesy of D. C. Herting, Distillation Products Industries.

[4] *d*-α-Tocopherol, type F-50, courtesy of D. C. Herting, Distillation Products Industries.

TABLE 2. *Ovine Blood Chemistries*[a] *as Related to WMD*

Year	Ration	Supplement	Blood Value	Ewes			Lambs		WMD Incidence		
				On trial	Prepartum	Postpartum	< 1 Week	2–6 Weeks	Gross	Micro	No. of Animals
1954–5	Control	None	E[b]	215±17	181±9			220±18[c]	0	0	8
			Ca[d]	10.1±.4	10.5±.2			11.0±.6			
			Mg[d]	2.4±.2	2.4±.1			2.0±.1			
			P[e]	5.4±.3	5.5±.4			10.1±.2			
	Basal	None	E	[f]	79±5	97±13		84±11	0	0	11
			Ca		9.5±.2	9.4±.3		10.8±.3			
			Mg		2.4±.1	2.7±.1		2.0±.1			
			P		5.5±.5	5.1±.7		7.9±.1			
1956–7	Control	None	E	61±6		55±7	88±36	42±5	0	0	15
			Ca	10.1±.1		10.6±.2	12.0±.7	11.2±.2			
			Mg	2.1±.1		2.3±.1	2.2±.2	1.9±.1			
			P	3.9±.2		3.8±.3	6.1±.1	7.0±.2			
			Hb[g]	14.2±.5		12.7±.4	11.7±1.0	11.6±.6			
	Basal	None	E	[f]		17±5	44±7	22±2	13	2	32
			Ca			10.6±.2	12.0±.4	12.2±.3			
			Mg			1.9±.1	2.3±.1	1.8±.1			
			P			4.0±.2	5.6±.1	7.5±.2			
			Hb			14.1±.4	13.1±1.0	9.3±.5			
1957–8	Control	None	E	208±15	103±7	119±11	63±17	74±11	0	1	18
			Ca	10.1±.1	9.5±.2	10.3±.2	11.9±.4	11.1±.1			
			Mg	2.4±.1	2.4±.1	2.1±.1	1.8±.1	1.8±.1			
			P	4.7±.1	3.7±.1	3.7±.2	7.0±.3	6.4±.3			
			Hb	12.4±.2	12.6±.2	12.3±.4	10.8±.5	13.3±.3			
	Basal	None	E	[f]	27±5	26±3	8±3	13±6	11	0	15
			Ca		9.9±.2	10.3±.2	12.1±.3	11.2±.4			
			Mg		2.3±.1	2.0±.1	1.7±.1	1.9±.1			
			P		3.4±.2	5.1±.2	5.7±.8	7.5±.3			
			Hb		13.6±.2	13.2±.4	10.4±1.0	10.9±1.0			
	Idem.	+ Oral E/ewes	E	[f]	57±9	20±9		20±5	16	0	20
			Ca		9.5±.4	10.4±.2		11.3±.5			
			Mg		2.2±.1	2.0±.1		1.7±.1			
			P		3.8±.5	4.5±.2		7.0±.5			
			Hb		13.9±.4	13.1±.3		11.0±.6			
	Idem.	+ Inj. E/ewes	E	[f]	72±4	53±10		33±5	9	2	15
			Ca		10.4±.2	10.7±.2		10.7±.3			
			Mg		2.0±.1	2.0±.1		1.8±.1			
			P		3.6±.1	4.8±.2		7.0±.4			
			Hb		12.9±.3	13.3±.4		10.3±.5			
	Idem.	+ Oral Se/ewes	E	[f]	24±4	12±4		22±3	0	3	16
			Ca		10.2±.2	10.3±.2		11.2±.1			
			Mg		2.4±.1	2.1±.1		1.8±.1			
			P			4.8±.2		7.3±.4			
			Hb		12.6±.3	13.1±.4		12.3±.3			
1958–9	Basal	None	E	63±3	13±2			28±9	16	3	20
	Idem.	+ oral E/lambs	E	[f]	[h]			95±14	0	5	17
	Idem.	+ Inj. Se/lambs	E		[h]			12±4	0	7	17
	Idem.	+ Oral Se/ewes	E		13±5			11±4	0	3	18
	Idem.	+ Oral Se + S/ewes	E		21±5			15±3	1	8	15

[a] Mean ± standard error. [b] Total tocopherols, μg/100 ml plasma. [c] Pasture accessible to dams. [d] mg/100 ml plasma. [e] mg/100 ml whole blood. [f] Control values are representative of all groups when going on trial. [g] g/100 ml whole blood. [h] Basal values are representative of all groups of ewes similarly treated.

and inorganic sulfur levels to those found in the forage which was fed to cows in 1955–56 and which had caused a very high incidence of WMD in their calves, in spite of an apparently adequate selenium content.

In terms of overcoming the incidence of WMD, there was little difference between prenatal or postnatal selenium treatments, nor vitamin E therapy of the lambs (Table 2). A distinct advantage of prophylaxis, however, is evident in the relative rates of gain (Table 4). In view of the relative indices of histopathological lesions recorded, it may be questioned whether the differences in weight gain should be attributed to growth stimulation by selenium or to variable sub-clinical persistence of the disease.

With regard to plasma tocopherol analyses, in spite of the prolonged elevation of the levels in lambs having received the vitamin and of extremely low values recorded for other lambs, no direct relationship with disease incidence is apparent.

The results of combined administration of selenite and sulfate to ewes are taken as evidence of interference with the prophylactic effect of selenium. This view is adopted because of a progressive increase in incidence over the course of the period of lambing. Of the first seven born, one case was encountered; six of the next seven were afflicted and the last born was a grossly evident case. In addition, the over-all gain to 6 weeks of age was greatly reduced in the lambs from ewes fed sulfate.

The rations of ewes of two additional groups were also supplemented, one with soybean oil meal and the other with linseed oil meal as practical sources of Factor 3 activity. Again little difference could be detected in the extent of WMD incidence in resulting lambs in these cases compared to those receiving selenium or vitamin E. The growth rates were, however, significantly better than all but those resulting from prenatal selenite administration.

TABLE 3. *Forage Analyses*

Ration	Year	Dry Basis						
		Ca, %	P, %	E[a], ppm	St[b], %	Si[c], %	Se, ppm	Mo, ppm
Basal: Alfalfa/Cows								
	1955–56	1.44	0.16	92	0.27	0.15	0.43	0.21
	1956–57	1.24	0.17	62	0.21	0.06	0.16	0.24
	1957–58	1.20	0.29	59	0.09	nil	0.11	1.95
Idem. Clover/Ewes								
	1956–57	1.34	0.18	28	0.13	0.04	<.05	0.24
	1957–58	1.35	0.24	27	0.17	0.04	<.05	0.30
	1958–59	1.47	0.16		0.17	0.04		
	1959–60	1.69	0.19		0.14	0.04		
Control:								
Grass hay[d]	1956–57	0.41	0.27	37	0.12	0.03		
Grass silage[d]	1956–57	0.28	0.22		0.12	0.04		
Alfalfa hay[e]	1957–58	1.19	0.17	55	0.20	0.06	0.50	3.86
Grass-clover hay[f]	1957–58	0.30	0.19	30	0.09	nil		

[a] Total tocopherols; >95% α tocopherol. [b] Total sulfur. [c] Inorganic sulfate sulfur. [d] Fed 50:50/est. dry matter to cows and ewes. [e] Fed alone to cows. [f] Fed 50:50 with alfalfa hay to ewes.

TABLE 4. *Rate of Growth in Lambs*

Treatment	Lamb Weights, lb.*		WMD Incidence		
	Birth	Gain to 6 weeks	Gross	Micro	No. of Animals
1958–59					
Basal None	9.9±.2	15.3±.9	16	3	20
Idem. + Oral E/lambs	9.0±.4	19.3±1.6	0	5	17
Idem. + Inj. Se/lambs	9.2±.3	20.8±1.4	0	7	17
Idem. + Oral Se/ewes	9.1±.5	27.2±2.9	0	3	18
Idem. + Oral Se + S/ewes	9.6±.5	21.6±1.9	1	8	15
1959–60					
Basal None	8.3±.5	20.1±1.9	6	6	21
Idem. + 50 mg Se/ewes	8.8±.8	23.0±1.0	0	2	12
Idem. + 0.1 ppm Se/ewes	9.1±.6	24.0±2.4	0	0	12

* Mean ± standard error.

TABLE 5. *2–6 Week Lamb Skeletal Muscle Analyses*

Treatment	mg/100 g Fat-Free Dry Matter				
	Ca	P	Mg	Na	K
1957–58					
Control[a]	27±4	574±63	123±5	371±15	1800±48
Basal[b]	4772–25	2391–558	332–93	1237–360	1773–464
Idem. + Oral E/ewes[b]	2978–12	1831–281	210–50	1227–294	1875–428
Idem. + Inj. E/ewes[b]	4521–15	2790–274	342–99	1168–255	1897–878
Idem. + Oral Se/ewes[a]	28±3	627±57	120±4	413±26	1638±42
1958–59					
Basal[b]	5180–34	3008–480	337–108	1122–253	1750–795
Idem. + Oral E/lambs[a]	47±2	780±12	135±5	331±33	1663±41
Idem. + Inj. Se/lambs[a]	58±3	750±16	165±5	443±32	1540±52
Idem. + Oral Se/ewes[a]	69±4	742±14	121±5	307±50	1511±75

[a] Group means ± standard errors. [b] Group ranges.

The experiment of 1959–60 was primarily designed to investigate factors influencing the prenatal course of WMD. The reference groups of ewes again received: a myopathy-inducing basal; and a supplement of 0.1 ppm Se throughout the latter two-thirds of gestation, plus 6 weeks post-partum. A third group of ewes was treated at the outset of the trial with a single aqueous oral drench of 50 mg selenium as sodium selenite, an amount approximating that received by the ration-supplemented group over the entire course of the trial.

The results shown in Table 4 indicate the single dose of the element to have been a practical equivalent of prolonged dietary exposure. This interpretation is qualified by less severe incidence of gross lesions in the basal group than in prior years. In turn this is reflected by lack of a significant growth response in the treatment groups, probably due to better performance of the 6-week basal lambs than in the previous year. It should be further noted that a single intramuscular dose at the rate of 30 mg Se/100 lb body weight had proven fatal to 11 of 12 ewes within 24 hr.

During the growing season of the 1959-60 basal forage, two 1-acre plots were set aside within the field. Of these, one was left untreated, while 600 lb gypsum was applied to the other. Accumulated hays produced by each of these areas served as the rations of two additional groups of ewes. The incidence of types of WMD lesions in lambs was then as follows: no gypsum—2 congenital, plus 7/necropsy, plus 1/histology; 600 lb/acre gypsum—4 congenital, plus 7/necropsy, plus 2/histology.

These equivocal results can possibly be explained by carryover effects of routine annual applications of 300 lb gypsum per acre of the field as a whole over 3 prior years. Reconcilation of these results with sulfur analyses awaits determination of the effect on selenium contents.

Reference again to Table 3 shows the present state of analyses with regard to the forages involved in the sheep trials. It would appear here that an actual deficiency of selenium exists. If sulfate interference is involved, the basic defect may reside in reduced plant uptake of selenium in the case of this crop. This is in contrast to the apparent unavailability of the element in the particular alfalfa used in the cattle experiments. Bovine versus ovine species differences may also be involved. Further attention to rather wide calcium-phosphorus ratios may be warranted. It is difficult, however, to attach specific significance to tocopherol values for the forage ration of the dams.

Certain analyses of muscle tissues related to WMD (3, 6) are shown in Table 5, but will be the subject of more comprehensive treatment elsewhere. The extreme, wide range in calcium and phosphorus contents of skeletal muscle among WMD-afflicted lambs precludes all but individual comparison to normal values. The extent to which selenium therapy, in general, and vitamin E treatment of the lambs only, results in remission of gross calcification is striking. The relative constancy of magnesium values irrespective of status with regard to WMD is noteworthy. Ratios of total sodium to potassium contents show rather wide variation between tissues of normal and diseased animals.

In summary, enzootic white muscle disease of lambs and calves has been reproduced under controlled conditions by feeding the dams during gestation on native hays from areas involved. The characteristic clinical symptom of erratic locomotion, as well as cardiac failure and the gross and histopathological lesions of skeletal and cardiac muscle degeneration and calcification have usually been observed in preweaning offspring.

Supplementation of the ration of the dams during gestation through to weaning with 0.1 ppm selenium, as sodium selenite, has consistently provided essentially complete protection against the disease, while vitamin E has not. Selenium and vitamin E therapy for newborn animals have been effective in obviating the grossly observable postmortem lesions, but not entirely those detectable microscopically. Limited trials indicate superiority of selenium over vitamin E therapy as measured by weight gains of the young.

Incidence of the disease has not always been associated with a demonstrable forage selenium deficiency and evidence suggests that sulfur antagonism can influence the biological availability of the trace amounts of selenium which are required.

The authors are indebted to Dr. H. D. Eaton, Storrs, Connecticut, and to Dr. K. F. Swingle, Bozeman, Montana, for provision of forage tocopherol analyses (8, 21, 25) and to Dr. P. R. Stout, Berkeley, California, for molybdenum and selenium analyses (10). We further acknowledge the efficient technical assistance of Mrs. Ann C. Esse, Mr. P. E. Williams, and of Mrs. Pauline Fleetwood. Preparation of the manuscript and participation in this symposium was made possible by a Public Health Service Senior Research Fellowship (JRS) and by the encouragement given by Dr. Klaus Schwarz, National Institutes of Health, Bethesda, Maryland, during its tenure.

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Biochemical interrelations between selenium and sulfur in plants and microorganisms[1]

ALEX SHRIFT

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THE NEW STATUS that selenium has gained as a respectable element is gratifying to those of us who have been studying its notorious aspects. The toxicity of this substance has had a long history, and ever since the first studies by Cameron in 1880 (4) on the incorporation of selenium into plants, this toxicity has been attributed to an interference with sulfur metabolism. Such a view is only natural, for both elements fall in Family VI of the periodic table, and more than any other pair of elements they possess similar physical and chemical properties. In the early 1930's, when selenium toxicity occupied the attention of many investigators, a good deal of experimental work supported this concept. The evidence from this era can be grouped into two general categories: *1*) the association of selenium with proteins and with the sulfur-containing fractions derived from hydrolyzed proteins; *2*) the rather specific competitive antagonism that was found to exist between the sulfate and selenate ions.

The results of these studies, at best, were only suggestive. In the final analysis they do not really prove, as was frequently implied, that organisms are unable to distinguish between sulfur and selenium analogues and consequently metabolize the latter along the same enzyme routes by which sulfur is used. But in the last decade, more direct evidence has accumulated to corroborate the early hypothesis. Today, we can claim with much greater assurance that: *a*) sulfur and selenium compounds compete with each other during the initial absorption process across the plant cell membrane; *b*) selenium analogues of organic sulfur metabolites occur in plants and microorganisms; *c*) selenium analogues actually are used as substrates by the enzymes of sulfur metabolites; *d*) proteins altered by the substitution of selenomethionine for methionine nevertheless function; *e*) organisms are able to adapt to selenomethionine and this adaptation involves changes in normal sulfur metabolism.

COMPETITION BETWEEN SULFUR AND SELENIUM COMPOUNDS DURING ABSORPTION

For many plants and several microorganisms cultured in the presence of sulfate and selenate ions, both growth and selenium content depend on the ratio of the two ions in the ambient nutrient solutions (23, p. 559; 28, p. 148). This sulfur-selenium relationship is not limited to the inorganic ion pair, however, but also exists for methionine and selenomethionine. Fig. 1 illustrates that the selenium concentration in cells of *Chlorella vulgaris*, whether the selenium is derived from selenate or from selenomethionine, is determined by the external concentration of the corresponding sulfur analogue. At any one exogenous level of the selenium compound, the concentration of cellular selenium falls with increase in exogenous sulfur; at a constant external S/Se ratio, even though the absolute levels are increased, the cellular selenium remains the same (21, 22). These data indicate that each pair of the structural analogues competes for a specific site in the cell membrane which is believed to form a complex with the substance prior to its transport into the cell.

Another system, although it does not involve a microorganism, also illustrates a possible competition between a sulfur metabolite and its selenium analogue during the absorption process. Leukemic leukocytes possess a much higher uptake of L-cystine than do normal leukocytes. In tests to see which of a variety of substances would decrease the uptake of S^{35} L-cystine, selenium-containing structural analogues of the metabolite proved to be most effective (Table 1). Both the D- and L-forms of selenocystine, as well as a phenyl substituted analogue were able to depress appreciably the uptake of the radioactive metabolite. Other structurally related compounds were ineffective (32).

The most direct analysis of S-Se antagonism during the absorption process is provided by the work of Leggett and Epstein who studied the kinetics of sulfate absorption by barley roots (10). Data on the rate of radiosulfate uptake under the influence of selenate were analyzed by the classical Michaelis-Menten analysis for enzyme action. In Fig. 2 are shown the absorption data, with the reciprocal of the absorption rate plotted against the reciprocal of the sulfate ion concentration (the substrate in this system). In the absence of selenate a straight line is obtained, except at very high sulfate concentrations, which shows that the rate of sulfate absorption is a function of its external concentration. In the presence of selenate the slope is greater, but the

[1] Investigations by the author were supported in part by grants from the National Science Foundation.

intercept remains the same. The interpretation given, therefore, is that the two ions compete for a site or carrier at the cell membrane, much as substrate and antimetabolite compete for the same enzyme. Other ions such as nitrate and phosphate were without effect. The Michaelis constant for radioactive sulfate was 0.19; for selenate, 0.15. These calculations show that the affinity with which the two ions are bound to the common binding site is of the same order of magnitude. But other factors besides the affinity for an absorption site must be instrumental in the uptake of these two ions, since earlier studies with a number of plants have shown that the internal S/Se ratio is often much greater than the external ratio (8).

OCCURRENCE OF ORGANIC SELENIUM COMPOUNDS

Once inorganic selenium compounds penetrate an organism is there evidence that they are metabolized into analogues of sulfur compounds? The search for such substances, despite many years of effort in a number of laboratories, has provided only a few instances for their existence. The very closeness of the chemical and physical properties makes separation of selenium from sulfur analogues difficult, and this fact, as well as the greater lability of some selenium compounds, has greatly hampered their isolation. With the advent of chromatographic techniques, however, some progress has been made.

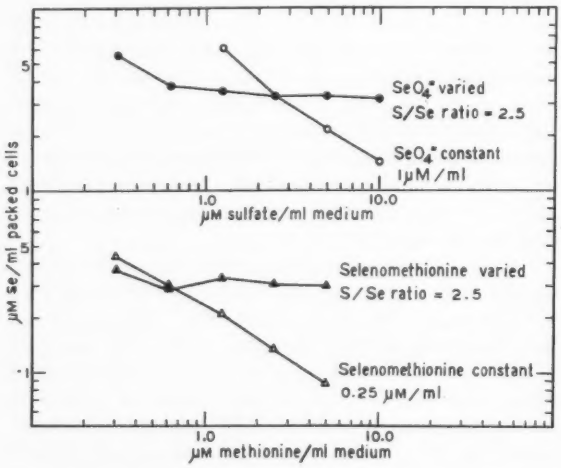

FIG. 1. *Top:* selenium concentrations in *Chlorella vulgaris* cells grown with sulfate and selenate (Shrift, 1954, 21). *Bottom:* cells grown with L-methionine and DL-selenomethionine (Shrift, 1954, 22).

TABLE 1. *Compounds Effective in Decreasing the Influx of S^{35} L-Cystine into Leukemic Leukocytes (Weisberger et al., 1956, ref. 32)*

Compound*	Uptake of S^{35}, % of Control†
1. L-Cysteine (unlabeled)	45
2. L-Selenocystine	45
3. D-Selenocystine	50
4. Phenyl selenocystine	50

* 1 μmole/ml blood used for preincubation prior to adding S^{35} L-cystine.

† % of Control

$$= \frac{\text{uptake after preincubation with compound}}{\text{uptake after preincubation with saline}}.$$

Table 2 summarizes the organic selenium compounds that have been reported in plants, microorganisms and animals. It should be emphasized that not one of the selenium-containing amino acids has been isolated in pure crystalline form, completely separated from its sulfur analogue, although Se-methylselenocysteine was obtained in a near pure state (29). Most of the identifications have been made by chromatographic comparison with synthetic selenium amino acids. But even with these techniques identification is often difficult, for selenium and sulfur analogues do not separate widely, if they are at all separable in any one solvent system. This difficulty can be overcome if the isolated unknown amino acid has been labeled with Se^{75}, for it can then be eluted and chromatographed with the synthetic seleno-amino acid as has been done for selenomethionine (31). The identification of selenocyst(e)ine presents special problems. The occurrence of a radioactive spot in the cysteine region of a protein hydrolystate chromatogram cannot of itself be considered evidence for the existence of selenocysteine. This substance is labile, its decomposition products have not been identified, and by analogy with what happens to cysteine during isolation procedures, mixed selenides or even selenosulfides can be expected to appear in the cyst(e)ine region on chromatograms.

Further complications arise when selenite is used in biosynthetic studies, since this form of selenium is known to react with sulfhydryl groups. The chemical nature of the resulting sulfur-selenium complexes and their behavior during extraction procedures has received relatively little attention (30). For these reasons, even radioactive areas on chromatograms of protein hydrolysates should be identified, not only by comparison with the positions of synthetic seleno-amino acids, but by other tests as well.

One of the immediate questions that arises from the reports of seleno-amino acids in animal tissues is the mechanism whereby animal cells reduce selenium from the oxidation level at which it is administered, usually as selenite or selenate, to the reduced level in amino acids. The animal cell has never been shown to possess enzymes for the reduction of sulfate to cysteine or methionine, although sulfur at the reduced level of sulfide can be utilized. If sulfur and selenium are really interchangeable, one is hard put to explain the reduction of selenium to its level in the amino acids. One plausible explanation is that the intestinal microflora is responsible for the observed traces of seleno-amino acids. Even in experiments in which inorganic selenium has been injected rather than fed (9, 12, 14, 18), 24 hr or more elapse before analysis is made, so that there is, conceivably, ample time for some selenium to reach the intes-

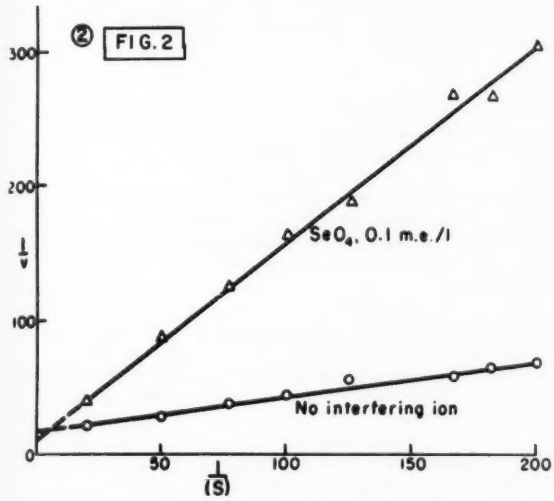

FIG. 2. Double reciprocal plot of the rate of absorption of S^{35}-sulfate by barley roots and the effect of selenate; the concentration of sulfate varied over the range 0.005 mEq/liter to 0.05 mEq/liter (Leggett and Epstein, 1956, 10).

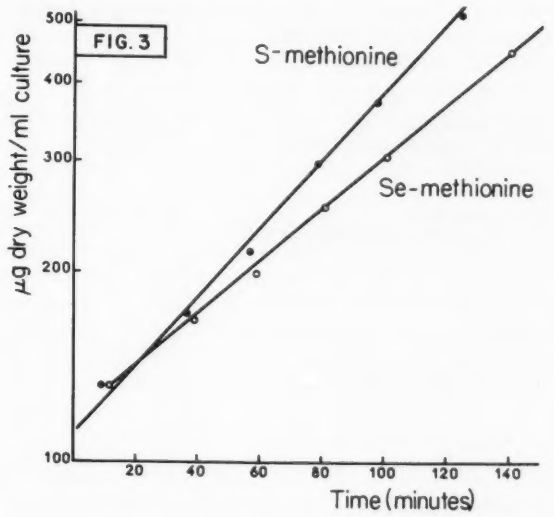

FIG. 3. Exponential growth of *Escherichia coli*, methionine-requiring mutant ML 304d, with 10^{-4} M DL-methionine or 10^{-4} M DL-selenomethionine (Cowie and Cohen, 1957, 5).

tinal flora, be metabolized, and returned to the bloodstream as amino acids.

To the list of organic selenium compounds summarized in Table 2 may be added several compounds that have been recognized as products when enzyme systems of sulfur metabolites act on the selenium analogue (Table 3).

SELENIUM ANALOGUES AS SUBSTRATES FOR ENZYMES OF SULFUR METABOLITES

The occurrence of selenium analogues of sulfur compounds cannot be considered proof that they are synthesized or metabolized by the same enzymes involved in sulfur metabolism. For proof of this sort, one must turn to more direct experiments with enzyme systems themselves.

Table 3 summarizes the enzyme systems known to function with either the sulfur or selenium analogues. An ATP sulfurylase, obtained from yeast, that catalyzes the formation of adenosine-5′-phosphosulfate (APS) and pyrophosphate from ATP and inorganic sulfate also acts on selenate. A nucleotide-bound Se^{75}-selenate could be detected in low yields by its electrophoretic position which was approximately the same as that of the APS. Under the experimental conditions of the enzyme assay, the APSe was less stable than was the APS (33).

A second enzyme system capable of acting on a selenium substrate is one that transfers methyl groups from methionine to a methyl accepto, This enzyme system consists of a methionine "activating" enzyme which first yields S-adenosylmethionine, and a methylpherase which then transfers the methyl group to a suitable methyl acceptor. Selenomethionine actually was a better substrate for the "activating" enzyme, prepared from yeast, than was methionine itself. In the system, the reaction was followed in two ways: by inorganic phosphate liberation and by formation of the adenosyl compound. The adenosylselenomethionine was precipitated as the trireineckate derivative and converted to the sulfate salt which was then incubated with guanidoacetic acid as methyl acceptor in the presence of pig liver creatine methylpherase. The adenosylselenomethionine was able to yield its methyl to form creatine almost as well as did the adenosylmethionine itself (15). Similarly, methylation enzymes in rat liver microsomes will transfer the methyl of adenosylselenomethionine to form choline and to methylate sulfhydryl groups (3).

Another instance in which selenomethionine can effectively replace methionine is that of an enzyme preparation which activates amino acids prior to their incorporation into proteins. With certain soluble enzyme fractions it is possible to show a reaction between the carboxyl group of the amino acid and ATP to give an amino acid adenylate and PP. Such an enzyme system, prepared from protoplasts of *Escherichia coli*, acted on selenomethionine equally as well as with methionine (16). Since this system is probably part of the mechanism whereby amino acids are incorporated into proteins, such a demonstration assumes added importance for the observations described below which show that functional altered enzymes that contain selenomethionine in place of methionine can be synthesized by at least two different organisms.

Clearly, these enzyme preparations, the sulfurylase, the methyl transferring systems, and the amino acid activating system, cannot distinguish between the sulfur and selenium analogue pair. Here is direct evidence

TABLE 2. *References to Naturally Occurring Organic Selenium Compounds*

Compound	Source		References
Se-methylselenocysteine $CH_3—Se—CH_2CH(NH_2)COOH$	*Astragalus bisulcatus*	Soil grown plants; aqueous extracts analyzed; 80% of total selenium as amino acid; isolated as crystalline solid with small amounts of S-methylcysteine.	(29)
Selenomethionine $CH_3—Se—CH_2CH_2CH(NH_2)COOH$	*Escherichia coli*	Grown with $Na_2Se^{75}O_3$; protein hydrolysates analyzed by column and two-dimensional paper chromatography; radioactive spot eluted from paper chromatogram and rechromatographed with synthetic selenomethionine; selenocysteine not found.	(31)
	Dog liver	Injected with $Se^{75}Cl_4$; protein hydrolysates analyzed by column and two-dimensional paper chromatography; radioactive spots close to but not coincident with synthetic seleno-amino acids, possibly due to large volumes of hydrolysates used.	(14)
	Corn and wheat seeds	Soil grown plants; protein hydrolysates subjected to one-dimensional paper chromatography; paper analyzed for selenium, and selenium locations compared with positions of synthetic seleno-amino acids.	(27)
	Sheep wool	Animal injected with $H_2Se^{75}O_3$; wool hydrolysates analyzed by column and paper chromatography.	(18)
Selenocysteine $HSe—CH_2CH(NH_2)COOH$	Dog liver	Animal injected with $Se^{75}Cl_4$; protein hydrolysates analyzed by column and two-dimensional paper chromatography; radioactive spots close to but not coincident with synthetic seleno-amino acids.	(14)
	Dog urine	Animal injected with $H_2Se^{75}O_3$; increase in urinary Se^{75} after animal fed bromobenzene; radioactivity associated with mercapturic acid fraction.	(12)
	Corn and wheat seeds	Soil grown plants; protein hydrolysates subjected to one-dimensional paper chromatography; paper analyzed for selenium, and selenium locations compared with positions of synthetic seleno-amino acids.	(27)
	Escherichia coli L-methionine-requiring mutant	Grown with Se^{75}-selenite; protein hydrolysate analyzed by two-dimensional paper chromatography; radioactive spot in region of cysteine; synthetic seleno-cysteine unavailable for comparison.	(5)
	Sheep wool	Animal injected with $H_2Se^{75}O_3$; wool hydrolysates analyzed by column and paper chromatography.	(18)
Selenotaurine $H_2NCH_2CH_2—SeO_3H$	Sheep bile	Animal injected with $H_2Se^{75}O_3$; taurocholic acid of bile separated on celite column.	(18)
Selenocoenzyme A $RNHCH_2CH_2—SeH$	Rat liver	Animal injected with $Na_2Se^{75}O_3$; coenzyme A identified chromatographically.	(9)
$C_{21}H_{42}N_6Se_2SO_{12}$ (Selenocystathionine and cystathionine, 2:1)	*Astragalus pectinatus*	Soil grown; aqueous extracts analyzed; isolated as a crystalline solid.*	(7)
Dimethylselenide $CH_3—Se—CH_3$	*Scopulariopsis brevicaulis* *Aspergillus niger*	Grown with Na_2SeO_4 or Na_2SeO_3; methyl groups originate from methionine.	(6)
	Rat	Injected with selenate; recovered from exhalations.	(13)
Factor 3 (structure as yet undetermined)	Obtained from a variety of natural materials such as pork kidney, brewer's yeast, and casein.		(20)†

* The mixture has been reported to separate into two spots on paper chromatograms (12); resolved into its two components, personal communication, Dr. K. Schwarz, National Institutes of Health, Bethesda, Md. † Also see K. Schwarz, this symposium.

TABLE 3. *Enzyme Systems That Use Selenium Analogues of the Sulfur-Containing Substrates*

Source	Reaction	Reference
ATP-sulfurylase		
Yeast	ATP + $SeO_4^=$ ⇄ Adenosine phosphoselenate + PP	(33)
Methionine "activating" enzyme		
Rabbit liver & yeast	ATP + selenomethionine → Se-adenosylselenomethionine + P + PP	(15)
Methylation		
Pig liver	Se-adenosylselenomethionine + guanidoacetic acid → creatine	(15)
Rat liver microsomes	Se-adenosylselenomethionine + microsomes → choline	(3)
Rat liver microsomes	Se-adenosylselenomethionine + 2-methoxyethanthiol → O,S-dimethylmercaptoethanol	(3)
Amino acid activating		
Escherichia coli protoplasts	ATP + selenomethionine → AMP-selenomethionine adenylate + PP	(16)

which confirms some of the speculations made by earlier investigators who were concerned with the toxicity of selenium compounds. Surprisingly, however, it has turned out that the ability of selenium compounds to replace sulfur metabolites need not be detrimental to an organism.

FUNCTIONAL ALTERED PROTEINS

A unique case of an organism able to use a selenoamino acid for growth is provided by the experiments of Cowie and Cohen with a methionine-requiring mutant of *E. coli* (5). This mutant was subcultured with selenomethionine through more than 100 generations in the complete absence of the "essential" methionine, with sulfate supplying the other sulfur needs of the cells. Growth, though somewhat slower than in the control (Fig. 3), was exponential, showing that enzymes necessary for growth and division of these cells functioned despite their altered state. Selenite, however, although it became associated with the proteins, could not replace sulfate for growth of the wild type cells. A widespread cellular substitution of selenium for sulfur, therefore, can be extremely toxic, whereas a partial substitution with selenomethionine seems compatible with continued cell function.

A somewhat comparable situation exists for the alga *Chlorella vulgaris*. With this species, cultured with sulfate to satisfy its sulfur needs, the presence of selenomethionine prevents the cells from dividing, but it does not prevent about half of them from growing into giant cells (22, 24, 25). During this period of growth, uncoupled from division, the cells are metabolically active, for there are marked increases in dry weight, in respiration, and in protein content (24). Hydrolysates of S^{35}-labeled proteins show the absence of methionine but the presence of sulfur amino acids in the cyst(e)ine region (Fig. 4). Apparently, for this organism, the selenium substitution is drastic enough to temporarily interfere with the division process but insufficient to prevent cell growth. There may possibly exist two classes of proteins in these cells, one class in which the sulfur of methionine is necessary for division; another class which can function in cell growth despite the alteration. In any event, these two cases show that it is possible for selenomethionine-altered proteins to function in cell growth of both organisms as well as in division of *Escherichia coli*. It would be of the greatest interest to know whether a partial substitution by other selenium compounds such as selenocysteine has such a remarkable biological consequence.

ADAPTATION TO SELENOMETHIONINE

The block to division caused by selenomethionine in *Chlorella vulgaris* is only temporary. Inhibited cultures are able to adapt and eventually resume divisions that give rise to progeny which cannot be distinguished from unadapted cells in most respects. Their appearance, size, weight, and protein content are identical with normal cells. Most important, their proteins once more contain methionine, its sulfur derived from sulfate (24).

The one feature that distinguishes adapted cells from untreated cells is their ability to divide without the period of uncoupled growth when subcultured in the presence of the analogue. The nature of this adaptation to selenomethionine has been investigated in some detail. Primary interest has been focused on the following aspects of the adaptation: *1*) whether it is a mass adaptation or the selection of a few selenomethionine-tolerant mutants; *2*) whether withdrawal of the analogue leads to reversal to the original state of sensitivity; *3*) the biochemical mechanism behind the adaptation, particularly in relation to the sulfur metabolism of the organism.

Fig. 5 illustrates that at the time when cell numbers begin to increase in a selenomethionine-inhibited population, there is a significant rise in the proportion of

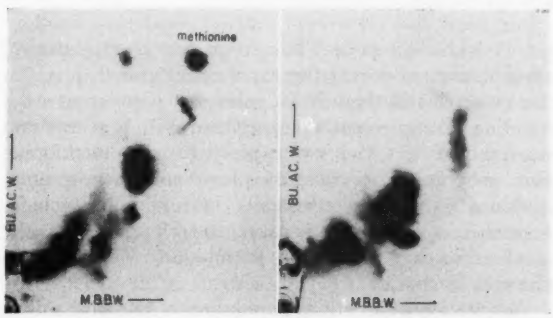

FIG. 4. Radioautographs of protein hydrolysates from *Chlorella vulgaris* cells grown with S^{35}-sulfate (*left*) or with S^{35}-sulfate + 3.1×10^{-5} M selenomethionine (*right*) (Shrift, 1959, 24).

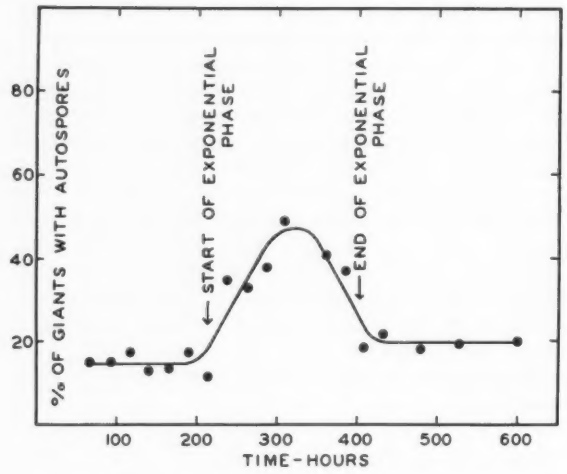

FIG. 5. Changes in proportions of giant *Chlorella vulgaris* cells containing autospores during culture with 3.0×10^{-5} M selenomethionine (Shrift et al., 1961, 25).

TABLE 4. *Failure of Chlorella vulgaris, Subcultured Six Times With Selenomethionine, to Deadapt After 22 Subcultures Without the Analogue (Shrift et al., 1961, 26)*

Adapted Series		Control Series	
No. of passages without seleno-methionine	Uncoupled phase after transfer to seleno-methionine*	+ Selenomethionine Duration of uncoupled phase*	Untreated Duration of lag phase*
	hr	*hr*	*hr*
1	29	130†	7
2	45	165†	14
13	26	160†	5
14	27	162	6
15	39	150	22
17	36	145†	23
22	32		14
	33±6‡		13±7‡

* Duration of uncoupled or lag phase calculated by extrapolating slope of exponential growth phase ($k = \ln N_2 - \ln N_1/t_2 - t_1$) to time axis, with inoculum size as base line. † Too few points to calculate k; duration of uncoupled growth estimated. ‡ Mean and 95% confidence interval.

giants with autospores. The frequency of reproducing giants increases during the exponential growth phase of the culture, and then drops when the population stops dividing. Complementary experiments on agar medium have shown that each cell exposed to selenomethionine first undergoes the enlarged phase and then resumes divisions to develop eventually into a visible colony. Selection of resistant mutants, therefore, is not the mechanism by which the population of *C. vulgaris* changes its character (25).

Adaptation of an entire population of microorganisms to a chemical is not a novel occurrence. In such systems the adaptation is invariably lost after several generations of growth away from the inducing agent. However, when selenomethionine was withdrawn from the adapted *C. vulgaris* population, the cells failed to revert. Table 4 summarizes the results of 22 subcultures, equivalent to about 220 generations, of an adapted population in the absence of selenomethionine. The duration of the lag remained unchanged and comparable to those of untreated cultures, in contrast to cells exposed to the analogue for the first time. Exposure to selenomethionine, therefore, has caused a stable, heritable change in an entire population of cells (26).

A logical place to search for a biochemical answer to this phenomenon appeared to be in the sulfur metabolism of these cells, rather than for induced enzymes able to metabolize selenomethionine, since induced enzymes should have disappeared after withdrawal of the analogue. Because the available evidence showed that the initial adaptation gave cells that contained methionine in their proteins, it looked as if the adapted cells were able to synthesize the amino acid from sulfate at a rate fast enough to effectively compete with and counteract the absorbed selenomethionine. As a working hypothesis, it was assumed that selenomethionine, in blocking the incorporation of methionine into proteins also caused the various intermediates between sulfate

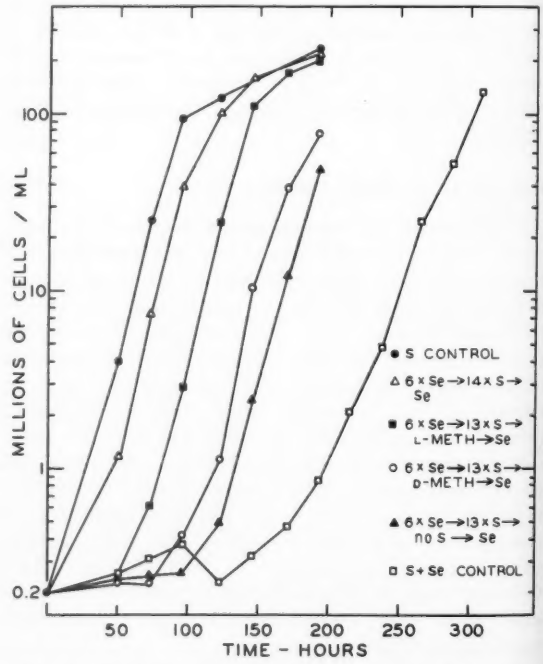

FIG. 6. Effects of cultural conditions on degree of adaptation in *Chlorella vulgaris*. Growth curves for adapted cells cultured with 3.1×10^{-5} M selenomethionine and 3.1×10^{-3} M sulfate after: △ 14 subcultures without the analogue; ■ 13 subcultures without the analogue and one subculture with L-methionine as only sulfur source; ○ 13 subcultures without the analogue and one subculture with D-methionine as only sulfur source; ▲ 13 subcultures without the analogue and one subculture without any sulfur. Normal control ●; cells cultured with selenomethionine for the first time □. (Shrift et al., 1961, 26).

and methionine to accumulate and to act as inducers for an increased synthesis of the enzymes responsible for their own further metabolism. The end result would then be a new steady state level for this particular metabolic route and a faster rate of methionine synthesis which could then compete with the absorbed selenomethionine. The actual inducing agents in this system would therefore be sulfate or its products rather than the selenomethionine itself. Experimentally, if this is the case, withdrawal of sulfate from the adapted cells should cause them to revert. Under sulfur starvation conditions, during which a few divisions occur, the sulfur intermediates should be depleted and the enzymes eventually return to their original levels, giving cells once more sensitive to selenomethionine (26).

Fig. 6 shows the results of a sulfur starvation experiment with adapted cells that had previously been subcultured 13 times in the absence of selenomethionine without any observed reversal. After the starvation treatment the sensitivity of the adapted cells to selenomethionine increased to the extent that it took about 108 hr for the cells to resume divisions rather than the 30 hr of the untreated adapted cells. Normal cells exposed to selenomethionine for the first time in this experiment had an uncoupled phase that lasted 162 hr. The graph also shows that subculture with D-methionine or with L-methionine, which these cells are able to use as their only source of sulfur, also causes partial reversal (26).

The deadaptation experiments suggest rather strongly that the initial adaptation does indeed involve a shift in steady state levels of the sulfate reducing enzyme pathway, and they fortify the idea that this particular biological activity of selenomethionine is intimately involved with changes in the sulfur metabolism of these cells.

PROBLEMS

Many problems exist in the field of selenium metabolism. One of the foremost is the question of selenium as an essential trace element for plants and microorganisms. For the plant physiologist three criteria must be met in order for a trace element to be considered essential: *1*) removal of the element must cause deficiency symptoms; *2*) application of the element, and no other, to the deficient plant must restore the plant to its healthy state; *3*) action of the element must be on the organism itself (1). This third criterion is a critical one. It must be established that the beneficial effect of a suspected trace element is not due to some secondary effect such as might stem from a change in the microbial flora associated with the organism, or perhaps to some change in the physiological balance of the other micronutrients.

The first two criteria were clearly met in 1938 by Trelease and Trelease (28, p. 16) who found that traces of inorganic selenium were needed for normal growth of a number of *Astragalus* species. Even the third criterion can be considered as having been met at that time, since it was already well established that these plants could convert inorganic selenium to organic forms. However, the purist might argue that, inasmuch as the chemical nature of the organic selenium was unknown, the conversion might have been a nonspecific association of the inorganic selenium with some plant component. This last objection has of course been overcome with the finding that most of the selenium in *Astragalus bisulcatus* exists as methylselenocysteine (29). Whether selenium at lower concentrations, in the range found effective for the prevention of deficiencies in animals, is essential for other plants, is still unknown.

In animal systems there are two ways to assess the role of a trace element. One way is to consider its effect on the animal as a whole with its integrated, intestinal flora. The other way is to consider the trace element in cellular metabolism. The dramatic effects caused by traces of selenium (19, 20) leave no doubt that the element functions in animal nutrition. But from the point of view of an essential role in the metabolism of the animal cell, the third criterion has not been wholly met. Until it has been proven that selenium functions in the cellular metabolism of the animal, or that the intestinal flora play no role in the observed physiological effects that result from selenium deficiency and selenium therapy, an essential role for the element in the animal cell can only be claimed with reservations.

A relatively untouched area of selenium metabolism is the ability of some *Astragalus* species and several other plants to accumulate selenium to high levels. This phenomenon, aside from purely biochemical aspects, presents an interesting aspect of plant evolution, for most other plants would be killed by these concentrations of selenium. A more detailed knowledge of what the accumulator plants do with the selenium they absorb should prove a valuable addition to the field of biochemical evolution whose subject matter is drawn primarily from work with animals and microorganisms.

With microorganisms there are two instances of a possible essential role for selenium. A brief report appeared in 1923 of an autotrophic bacillus isolated from soil and able to oxidize elemental selenium to selenic acid, much as *Thiobacillus thiooxidans* oxidizes sulfur to sulfuric acid (11). Further reports on this work, unfortunately, have not been published. A selenium requirement for synthesis of the adaptive enzyme, formic dehydrogenase, by a strain of *Escherichia coli* has been reported (17). The simultaneous presence of selenite (3×10^{-8} to 1×10^{-7} M), molybdate (1×10^{-8} M) and iron was essential, but since normal growth occurred without the first two ions, it is difficult to state that selenite is essential for the organism, although it may be required for synthesis of the particular adaptive enzyme.

A persistent problem in the field of selenium metabolism is whether organic selenium compounds exist in addition to the ones already discussed. If selenium and sulfur are really interchangeable then there must be a host of organic forms such as analogues of biotin, thia-

min, lipoic acid, to mention just a few important metabolites. And there is then the question, will these compounds exert extraordinary biological effects such as the growth uncoupling caused by selenomethionine in *Chlorella vulgaris* or the replacement of methionine in the methionine-requiring mutant of *Escherichia coli*?

Lastly, there are indications that inorganic selenium compounds may exert their toxic effects through other mechanisms. Arsenic and phosphorous, for example, have been found to antagonize selenium inhibition of yeast respiration (2); selenite itself is a strong oxidizer of sulfhydryl groups, a reaction that must be taken into consideration when this form of selenium is used in toxicity studies.

CONCLUSIONS

Selenium compounds exert powerful biological effects, and it is clear that these effects are intertwined with sulfur metabolism. From the very first step that we recognize in the metabolism of an inorganic substance by a plant or microorganism, that of its transport across the cell membrane, to its enzymatic conversion, selenium and sulfur are interchangeable. To this extent at least, the older ideas of selenium toxicity have been verified. But it has also become apparent that not all forms of selenium need be toxic. Not only is it possible for an organic selenium analogue to support some or all of a cell's functions if the remainder of the sulfur needs are satisfied, but it is possible for an organism to adapt and to thrive in the presence of a selenium compound that originally was inhibitory. Our ideas of the element will have to be revised, not only because of its newly discovered role in animal nutrition, but also because it is no longer as formidable an element for plants and microorganisms as we were led to believe.

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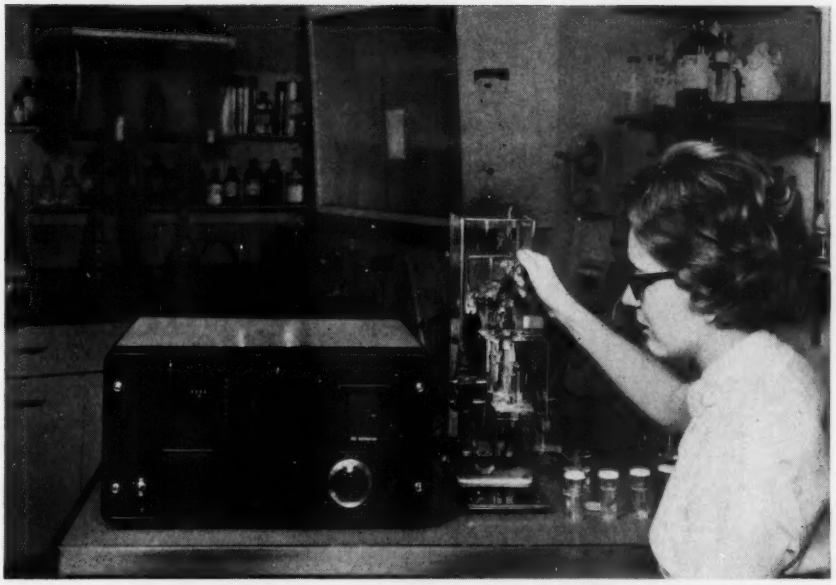
FINANCIAL REPORTS

EXHIBIT A. Balance Sheet—December 31, 1960

Assets:	
Cash in banks	$149,609
Accounts receivable	112,717
Building and loan deposits	55,368
Inventory of books and supplies	8,572
Prepaid expenses	16,962
Land, buildings and equipment (less accumulated depreciation of $56,410)	176,920
Trust fund investments at cost (market value $27,600)	16,482
Total Assets	$536,630
Deduct—Liabilities:	
Accounts payable and accrued expenses	$ 63,316
Mortgage payable	33,000
Deferred income	236,643
Total Liabilities	$332,959
Net Assets, Representing Fund Capital (per Exhibit B Below)	$203,671

EXHIBIT B. Statement of Income and Expenses and Fund Capital for Year Ended December 31, 1960

Income:	
FEDERATION PROCEEDINGS:	
Assessments on member societies	$ 23,604
Subscriptions, advertising, reprints, etc.	99,400
Annual Meeting	171,716
Other meetings	71,658
Placement information service	10,679
Grants and government contracts	360,448
Rental income	8,251
Dividends and other income	7,094
Total Income	$752,850
Expenses:	
Salaries, wages, Social Security, group insurance, and pension	$234,008
Printing and engraving	114,845
Advertising and exhibit commissions	32,999
Projection and public address services	6,938
Professional and other personal services	39,257
Rentals and facilities equipment	27,751
Grants and fellowships	118,435
Operation and maintenance of buildings, grounds, and equipment	28,096
Supplies and duplicating	37,733
Communications, shipping, and travel	52,271
Other expenses and supplies	8,743
Total Expenses	$701,076
Excess of Income Over Expenses	$ 51,774
Fund Capital:	
Balance, December 31, 1959	151,897
Balance, December 31, 1960	$203,671



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6,8-Dithiol-octanoic acid amide (reduced d,l-α-lipoamide)
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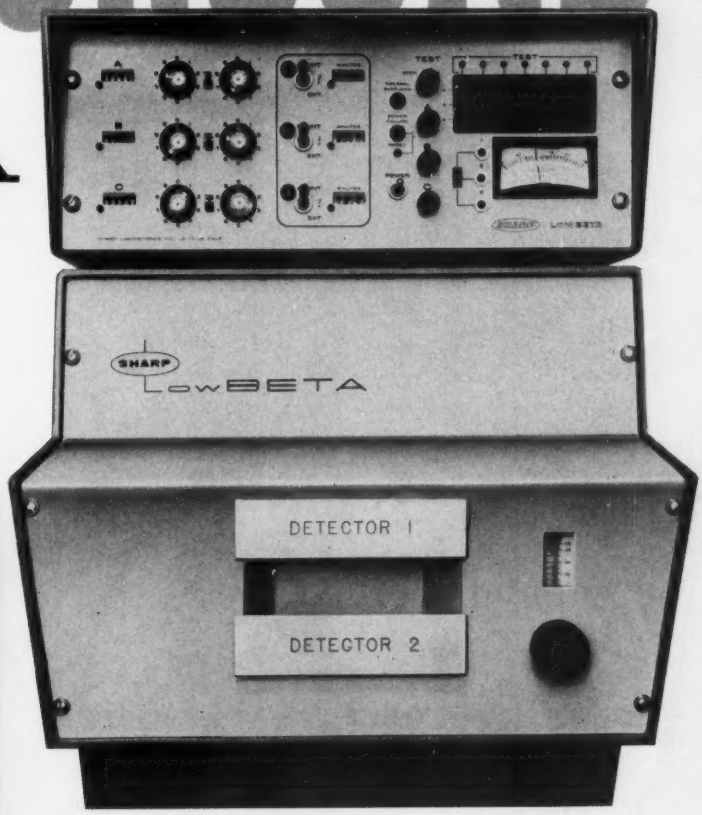

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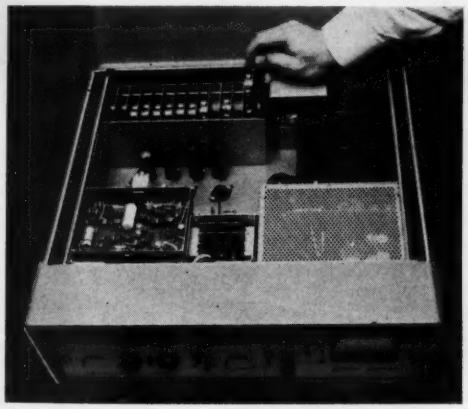

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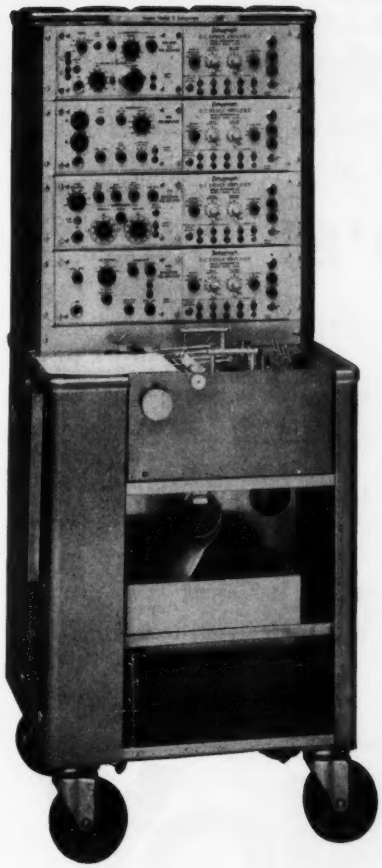

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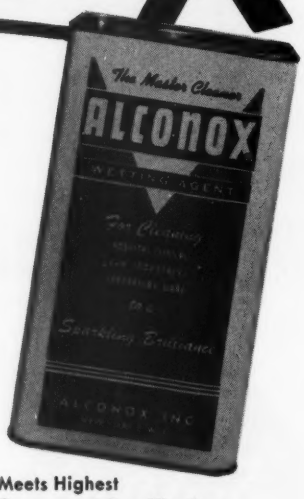

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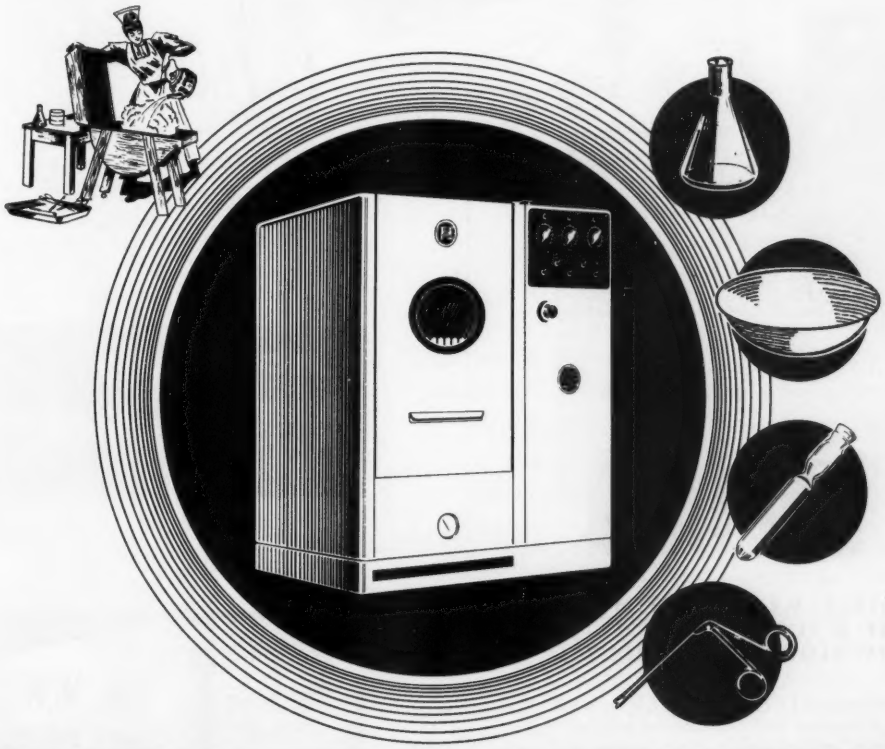

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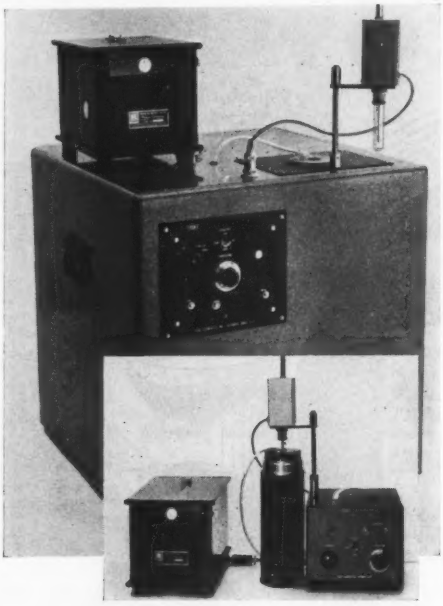

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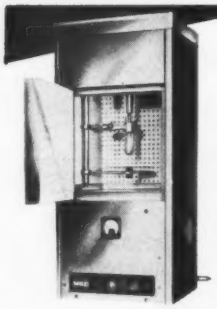

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News & Notes

NOTES ON THE FORTY-FIFTH ANNUAL MEETING OF THE FEDERATION

Atlantic City, New Jersey, April 10–14, 1961

Registered attendance at the forty-fifth Annual Meeting increased some 1,500 over the 1960 registration to a total of 12,567. Of this total 2,852 were members of the Federated Societies; 6,349 non-members; 1,420 graduate students; 345 guests; 71 press; 1,151 exhibitors; 190 guests of exhibitors; and 189 ladies' program.

Scientific sessions were held in Convention Hall, and in the Ambassador, Dennis, Ritz-Carlton, Shelburne, and Traymore Hotels. In all, 2,815 papers were presented in 270 scientific sessions. There were 43 Intersociety Sessions.

There were 14 Society symposia and special sessions. The American Society for Experimental Pathology, host Society for the Meeting, sponsored the Joint Session on Approaches to Study of Disease on Tuesday evening, April 11. Dr. Cyrus C. Erickson, Chairman of the Federation Board, presided.

The form of the Motion Picture Session was revised this year. A total of 14 motion pictures were presented in continuous theater style during three full days of the Meeting, April 11, 12, and 13.

Four scientific awards were presented during the Annual Meeting. The *Borden Award in Nutrition* consisting of $1,000 and a gold medal presented annually by the Borden Company Foundation was awarded to *Dr. Klaus Schwarz*, Chief of the Section on Experimental Liver Diseases, National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health. The *Osborne and Mendel Award* of $1,000 presented by the Nutrition Foundation, Inc. was given to *Dr. Max K. Horwitt*, Director of the Research Laboratory at the Elgin State Hospital, Elgin, Illinois. *Dr. Donald B. Hackel*, Duke University Medical Center, received the 5th annual *Parke-Davis Award* consisting of $1,000 and a bronze medal for outstanding work in the field of pathology. *Dr. Don W. Esplin*, Assistant Research Professor of Pharmacology, University of Utah College of Medicine, received the *John J. Abel Award* of $1,000 presented by Eli Lilly & Co.

During the week of the Annual Meeting, 117 special meetings, dinners, luncheons, breakfasts, and social hours were held. Councils of the six member Societies, the Federation Board, and the Federation Advisory Committee all conducted meetings, and all Societies held business meetings.

An active Press Room was managed by Dr. Sidney S. Negus, Federation Director of Public Information for the Annual Meeting, with the assistance of the Federation Public Information Committee headed by Dr. Robert B. Jennings.

The latest in scientific books, apparatus and equipment, pharmaceuticals, chemicals and food products were among the 236 industrial and 19 institutional exhibits on display in Convention Hall.

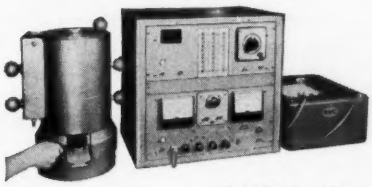
1962 Annual Meeting

The next Annual Meeting of the Federation will be held in Atlantic City, with registration opening on Friday, April 13. It is anticipated that scientific sessions will run for six days, from Saturday, April 14, through Thursday, April 19, 1962.

Continued on page lxxviii

NUCLEAR INSTRUMENTS

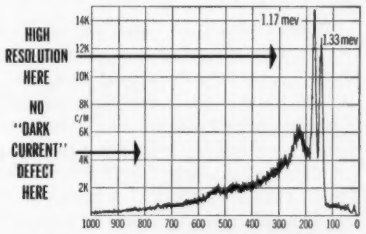
This Gamma Spectrometer Ends "Dark Current" Defect; Provides Direct Peak Comparison and Better Resolution from 1 kev to 6 mev.



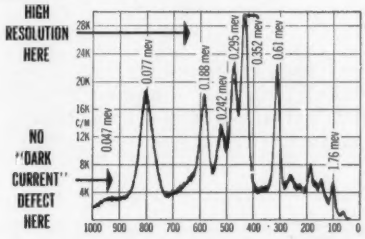
Model GSS-1—$4,180*

The NMC Model GSS-1 gamma spectrometer combines in one chassis a regulated high voltage supply as a part of the counting ratemeter autoscan system . . . all-transistorized probe amplifier and spectrometer circuits . . . and either solid or well-type crystals. It's the only system that eliminates the "dark current" defect, analyzes emitters from 1 kev to around 6 mev, and permits direct peak comparison of emitters using semi-automatic peak integration. Graph paper supplied is calibrated in mev.

Actual Spectrograms Made with a GSS-1



Actual spectrogram of Cobalt-60 made with a GSS-1 gamma spectrometer system. Window No. 3. Time constant: 2 sec.

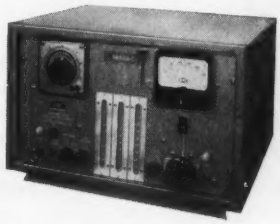


Radium, after four runs over 20 hour period without instrument adjustment. Range 30,000 C/M. Time constant: 3 sec.

Detect and Measure α, β and γ Four Ways with This 3-Unit All-Purpose Laboratory—1 . . . or 1+2 . . . or 1+3 . . . or 1+2+3

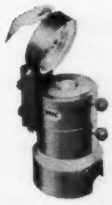
1 PROPORTIONAL COUNTING SYSTEM
PC-3A—$1395.00*

The only instrument that counts every alpha and beta emitted in a 2 pi angle from prepared samples. Precisely separates absolute amounts of alpha and beta from any dry sample. Ideal for C-14, Ca-45, P-32 and H-3. Due to high yield, counting time may be reduced 80% to 95%. High voltage and scaling systems operate with US-1 and WSC-1 shown below.



2 UNIVERSAL SHIELD
US-1A—$395.00*

Detects gamma and beta-gamma activity in prepared flat samples. Also accepts gamma scintillation detector, mylar window GM flow detector, and strip chromatograph scanner. Operates with PC-3A.



3 WELL-TYPE COUNTER
WSC-1—$995.00*

Background less than 140 cpm. Detects low-level gamma activity in test tube and flat type samples. All-transistorized amplifier. Works with any scaler. Operates with the PC-3A.

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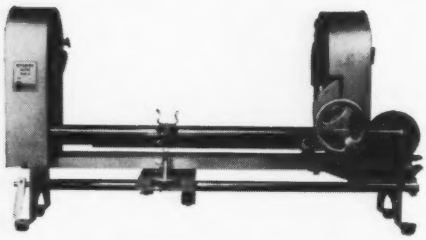

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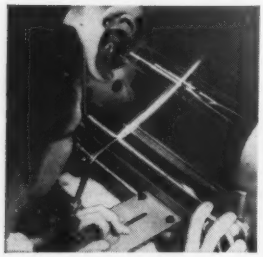

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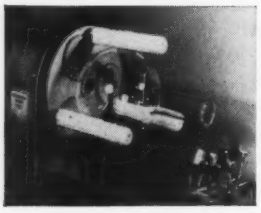

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Continued from page lxxvi

Meetings

The *Gordon Research Conferences* on *Food and Nutrition* will be held August 21–25, 1961, at Colby Junior College, New London, New Hampshire. The Conferences on *Cancer* will meet at the same location, August 28–September 1, 1961. Attendance at the Conferences is by application. Requests for attendance at the conferences, or for information, should be addressed to W. George Parks, Director, Colby Junior College, New London, N. H.

Boston will be the site of the *12th Annual Animal Care Panel Meeting*, September 27, 28, 29, 1961. The ACP is an organization of individuals professionally engaged in the production, procurement, care, management and use of laboratory animals and of institutions interested in these areas of research and animal care. The American Board of Laboratory Animal Medicine, the Laboratory Animal Breeders' Association and the Allied Trade Association will hold simultaneous sessions at the meeting.

The *11th Annual Instrument Symposium and Research Equipment Exhibit* will be held October 9 through 12, 1961, at the National Institutes of Health, Bethesda, Maryland.

Third Symposium on Cinefluorography. The staff of the Department of Radiology at the University of Rochester regrets that it was not possible to hold this Symposium in the Spring of 1961 as originally announced. Instead, the meeting will be held in Rochester, New York, December 1 and 2, 1961. This announcement is an invitation for the submission of scientific papers dealing with any facet of the technical or applied aspects of motion picture radiography. Please address inquiries or applications to: Stanley M. Rogoff, M.D., Division of Diagnostic Radiology, University of Rochester Medical Center, Rochester 20, New York.

Awards

Several Federation members have been honored as recipients of *Borden Awards for 1960* which are given in recognition of outstanding achievement by scientists of the United States and Canada.

The American Physiological Society claims two members, *Dr. Henry Hugh Dukes* and *Dr. Robert F. Pitts.* The former received his Award "in recognition of his long-continued studies in the general area of animal physiology, with particular reference to the physiology of digestion in ruminants." Dr. Pitts was recognized for his "fundamental contributions to an understanding of renal tubular functions."

Dr. Marian E. Swendseid, a member of both the American Society of Biological Chemists and the American Institute of Nutrition was named by the American Home Economics Association for the 1960 Borden Award for her research in vitamin and protein nutrition.

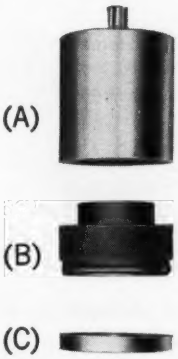
The American Institute of Nutrition was represented by two additional members: *Dr. Norman L. Jacobson,* selected by the American Dairy Science Association for his notable research contributions in dairy animal nutrition; and *Dr. Harold E. Harrison,* chosen by the American Academy of Pediatrics for his fundamental research in the physiology of vitamin D and its role in the regulation of the transport of calcium and phosphorous.

The Board of Directors of the Passano Foundation announces that *Dr. Owen H. Wangensteen* has been selected as the recipient of the $5000 *Passano Award for 1961.* Dr. Wangensteen is a member of the American Physiological Society and the American Society for Experimental Pathology. The Passano Foundation has as its sole purpose the encouragement of medical science and research, particularly that having a clinical application. It is sustained by annual contributions from The Williams & Wilkins Company, publishers of books and periodicals in medicine and the allied sciences.

Continued on page lxxx

New Tracerlab FLOW COUNTERS feature

Mono-Mol (<125 μg/cm²) Windows... Exceptional Plateau Characteristics... Plug-In Construction



Exploded view of the FD Series Flow Counter showing (A) Counter Shield (B) Plug-in Flow Counter (C) Snap-On Window.

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The basic counters are available in 1″ or 2″ models and both operate in the geiger region with standard Tracerlab G-1 gas, or in the proportional region with argon methane G-2 Gas, pure methane or natural gas.

Construction is all plastic to minimize background and cathodes are deposited stainless steel.

A glance at the operating specifications of the new Tracerlab Flow Counters will show you why these low cost, long lasting, extremely efficient units belong in your sample counting system.

SPECIFICATIONS THIN WINDOW FLOW COUNTERS

Model No.	Operating Mode	Gas	Required System Sensitivity	Operating Voltage	Background	Plateau Length	Plateau Slope over entire range
FD-1*	Geiger	G-1	.25V	1450V ± 150	<12 c.p.m.	300V	<2%/100V
FD-2**	Geiger	G-1	.25V	1450V ± 150	<30 c.p.m.	300V	<2%
FD-1	Prop.	G-2	α 10 mv	2200V ± 500	α < 6 c.p.h.	1000V	<2%
			β 1 mv	2300V ± 200	β <12 c.p.m.	400V	<2%
FD-1	Prop.	Pure Methane	α 10 mv	3500V ± 500	α < 6 c.p.h.	1000V	<1%
			β 10 mv	4500V ± 500	β <12 c.p.m.	1000V	<1%
FD-1	Prop.	Natural Gas	α 10 mv	4000V ± 500	α < 6 c.p.h.	1000V	<2%
			β 10 mv	4700V ± 300	β <12 c.p.m.	600V	<2%
FD-2	Prop.	G-2	α 10 mv	2200V ± 500	α <12 c.p.h.	1000V	<2%
			β 1 mv	2300V ± 200	β <30 c.p.m.	400V	<2%
FD-2	Prop.	Pure Methane	α 10 mv	3000V ± 500	α <12 c.p.h.	1000V	<1%
			β 10 mv	4500V ± 500	β <30 c.p.m.	1000V	<1%
FD-2	Prop.	Natural Gas	α 10 mv	4000V ± 500	α <12 c.p.h.	1000V	<2%
			β 10 mv	4700V ± 300	β <30 c.p.m.	600V	<2%

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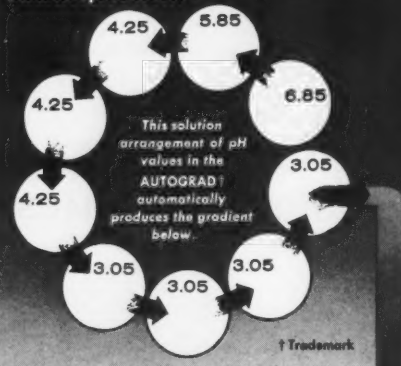

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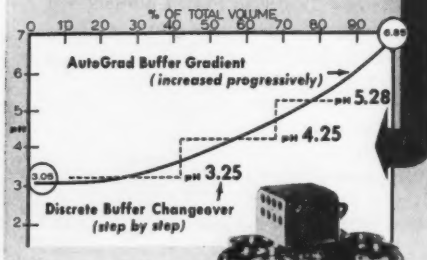

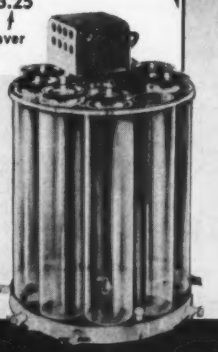

Developed at the National Cancer Institute, National Institutes of Health, U. S. Department of Health, Education, and Welfare, Public Health Service, Bethesda, Maryland.

***Procedure developed by *Karl A. Piez, National Institute of Dental Research, National Institute of Health.*

*Reference: *"Variable Gradient Device for Chromatography", E. A. Peterson and H. A. Sober, Anal. Chem., Vol. 31, No. 34, May 1959.*

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Continued from page lxxviii

Books and Journals Received

ABSTRACTS OF JAPANESE MEDICINE. Editors: T. Yoshida and J. Huizinga. Amsterdam: Excerpta Medica Foundation. Monthly publication, subscription rate $30/yr.

ADRENERGIC MECHANISMS. G. E. W. Wolstenholme and Maeve O'Connor, Editors for the Ciba Foundation. Boston: Little, Brown & Co., 1960. 632 pp. $12.50

AEROSPACE MEDICINE AND BIOLOGY, An Annotated Bibliography. Arnold J. Jacobius and others. Science and Technology Division, The Library of Congress, Wash. D.C. Volume IV, 1955 Literature, 1961

ANIMAL PARASITES IN MAN. N. H. Swellengrebel and M. M. Sterman. Princeton, N. J.: D. Van Nostrand Co., Inc., 1961. 652 pp. $12.00

ANNUAL REVIEW OF PHYSIOLOGY—Vol. 23. Editor: Victor E. Hall. Palo Alto, Calif.: Annual Reviews, Inc., 1961. 674 pp. $7.00 prepaid (USA) $7.50 prepaid (elsewhere)

BASIC PHYSIOLOGY. Fred E. D'Amour. Chicago: University of Chicago Press, 1961. 642 pp. $7.95

BLOOD AND OTHER BODY FLUIDS. Analysis and Compilation by Philip L. Altman, edited by Dorothy S. Dittmer. Wash., D.C.: Committee on Biological Handbooks, Federation of American Societies for Experimental Biology, 1961. 540 pp. $10.00

BULLETIN OF THE MEDICAL LIBRARY ASSOCIATION, Vol. 49, No. 1 Pt. 2, The National Library of Medicine Index Mechanization Project. Wash., D.C.: National Library of Medicine, 1961. 96 pp. $1.75 dom. $2.00 foreign

CARDIAC SURGERY 1960–1961. Edited by Charles P. Bailey. Phila.: F. A. Davis Co., 1960. 179 pp. $5.00

CLINICAL DISTURBANCES OF RENAL FUNCTION. Abraham G. White. Phila: W. B. Saunders Co., 1961. 468 pp. $10.50

DIFFERENTIAL DIAGNOSIS OF THE ELECTROCARDIOGRAM. Sidney R. Arbeit, Ira L. Rubin, Harry Gross. Phila.: F. A. Davis Co., 1960. 212 pp. $10.50

ELECTRICAL ACTIVITY OF SINGLE CELLS. Edited by Yasuji Katsuki. Tokyo: Igaku Shoin Ltd., 1960. 312 pp.

ENZYMOLOGY IN THE USSR. A Review of the Literature. Chester W. DeLong. U.S. Dept of Health, Education, and Welfare, Public Health Service, 1960. 189 pp. Available from Supt. of Documents, U.S. Gov't. Printing Office, Wash. 25, D.C., $0.65 (PHS publ. no. 782)

ESSENTIAL HYPERTENSION, An International Symposium, sponsored by CIBA. Edited by E. Buchborn and K. D. Bock. Berlin: Springer-Verlag, 1960. 382 pp.

EXPLORATIONS INTO THE NATURE OF THE LIVING CELL. Robert and Edward L. Chambers. Cambridge, Mass.: Harvard University Press, 1961. 352 pp. $8.00

HANDBOOK OF PHYSIOLOGY, 43rd Ed. R. J. S. McDowall. Phila.: J. B. Lippincott Co., 1961. 759 pp. $12.50

INDEX—HANDBOOK OF CARDIOVASCULAR AGENTS, Vol. 2, Pts I & II. Isaac D. Welt. Wash., D.C.: National Academy of Sciences-National Research Council, 1960. 1568 pp. $15.00

INTEGRATED PRINCIPLES OF ZOOLOGY, 2nd Ed. Cleveland P. Hickman. St. Louis: The C. V. Mosby Co., 1961. 972 pp. $7.75

JOURNAL OF ATHEROSCLEROSIS RESEARCH. International Journal of Fundamental Research in Atherosclerosis and Related Subjects. Elsevier Publishing Co., Amsterdam, Vol. 1, No. 1—January/February 1961. Bi-monthly, $17.50 per year.

MEDICAL NURSING. Edition 6. Edgar Hull and Cecilia M. Perrodin Phila.; F. A. Davis Co., 1960. 798 pp. $6.95

METABOLIC EFFECTS OF ADRENAL HORMONES. G. E. W. Wolstenholme and Maeve O'Connor, Editors for the Ciba Foundation. Boston: Little, Brown and Co., 1960. 109 pp.

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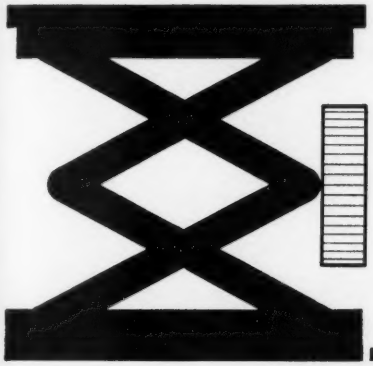

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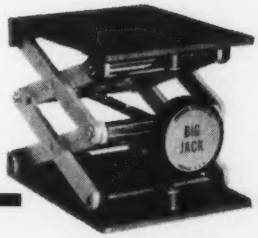

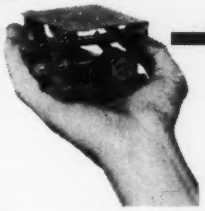

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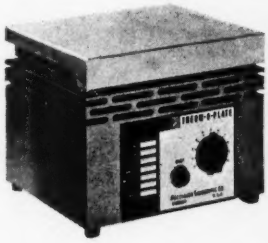

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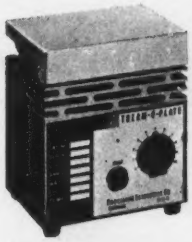

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